Role of Cellular Casein Kinase II in the Function of the Phosphoprotein (P) Subunit of RNA Polymerase of Vesicular Stomatitis Virus*

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The phosphorylation of the P protein of vesicular stomatitis virus by cellular casein kinase II (CKII) is essential for its activity in viral transcription. Recent in vitro studies have demonstrated that CKII converts the inactive unphosphorylated form of P (P0) to an active phosphorylated form P1, after phosphorylation at two serine residues, Ser-59 and Ser-61. To gain insight into the role of CKII-mediated phosphorylation in the structure and function of the P protein, we have carried out circular dichroism (CD) and biochemical analyses of both P0 and P1. The results of CD analyses reveal that phosphorylation of P0 to P1 significantly increases the predicted a-helical structure of the P1 protein from 27 to 48%. The phosphorylation defective double serine mutant (P59/61), which is transcriptionally inactive, possesses a secondary structure similar to that of P0. P1, at a protein concentration of 50 μg/ml, elutes from a gel filtration column apparently as a dimer, whereas both P0 and the double serine mutant elute as a monomer at the same concentration. Interestingly, unlike wild-type P1 protein, the P mutants in which either Ser-59 or Ser-61 is altered to alanine required a high concentration of CKII for optimal phosphorylation. We demonstrate here that phosphorylation of either Ser-59 or Ser-61 is necessary and sufficient to transactivate L polymerase although alteration of one serine residue significantly decreases its affinity for CKII. We have also shown that P1 binds to the N-RNA template more efficiently than P0 and the formation of P1 is a prerequisite for the subsequent phosphorylation by L protein-associated kinase. In addition, mutant P59/61 acts as a transdominant negative mutant when used in a transcription reconstitution assay in the presence of wild-type P protein.

The RNA-dependent RNA polymerase of vesicular stomatitis virus (VSV) consists of two proteins: the large protein L (241 kDa) and the phosphoprotein P (29 kDa). Together, these proteins are needed to transcribe the linear, single-stranded viral RNA genome of negative polarity, which is tightly wrapped with the nucleocapsid N protein (N-RNA template) (1, 3). Genetic and biochemical studies have suggested that the L protein encodes all the basic transcription activities, whereas the P protein appears to be an RNA virus transcription factor (1, 2, 7) with properties similar to many well studied eucaryotic transcription factors/activators (31). The P protein contains a-helical coiled structure and is highly acidic, with Asp and Glu residues constituting one-third of the first 100 amino acid residues in the N-terminal half (domain I) of the polypeptide (17, 18). The acidic domain is also phosphorylated by cellular protein kinase (14, 15). The possible contribution of the N-terminal acidic domain I in the function of P protein seems to transactivate the L protein for transcription similar to those observed for eucaryotic acidic transactivators (30–32). The C-terminal end, on the other hand, serves as the binding site for the L protein (domain II) and the N-RNA template (domain III) (8, 13). Initial studies of P protein isolated from virions or infected cell extract indicated that it exists in a variety of phosphorylated states and that this phosphorylation event was important for the transcriptional activity of L (10–12). Recently we have shown that cellular protein kinase, casein kinase II (CKII), is directly involved in phosphorylating the P protein at serine residues 59 and 61 in domain I (6); activation of P protein occurs following this initial phosphorylation event. Two additional sites at the C-terminal domain (domain II) are also phosphorylated by an L protein-associated kinase at serine residues 236 and 242, as determined previously by mutational analyses of recombinant P protein (16).

The role of cellular CKII in the phosphorylation of P protein was demonstrated in vitro primarily by using the unphosphorylated form of P protein obtained by expression of the P gene in Escherichia coli (4). Two forms of P protein (NJ serotype) were shown to be involved in the activation process: a partially phosphorylated intermediate (P1) and a fully phosphorylated form (P2). Cellular CKII phosphorylated bacterially expressed P0 and converted it into P1, but not to P2, demonstrating that P1 is the end product of cell kinase-mediated phosphorylation. A highly purified L protein preparation failed to phosphorylate P0 but phosphorylated P1 to produce P2. Thus, a cascade phosphorylation pathway was proposed in which a sequential phosphorylation step occurred as P0 → P1 → P2, leading to the activation of the P protein (5, 6). Thus, it seems that phosphorylation of the P protein by CKII is the first biosynthetic event in the infected cell that possibly leads to a conformational change in the P protein such that domain II becomes accessible to L-kinase. However, the precise role of CKII-mediated phosphorylation in the structure and function of the P protein remains unclear.

In an attempt to understand the phosphorylation pathway and the role of phosphorylation in P function, we have carried out structure-function analyses of the P protein in more detail using various P mutants. Here, we demonstrate that phosho-
ylation by cellular CKII induces a profound increase in the predicted α-helical structure and in the apparent dimerization of the P protein. Determination by gel filtration analysis. In addition, phosphorylation facilitates the binding of P to the N-RNA template as well as subsequent phosphorylation by L-associated kinase. We have characterized two P phosphorylation mutants which require higher concentration of CKII for L-associated kinase. We have characterized two P phosphorylation mutants which require higher concentration of CKII for transactivation mutants which require higher concentration of CKII for their optimal phosphorylation leading to activation and concomitant alteration of structure of the P protein.

**EXPERIMENTAL PROCEDURES**

Materials—All enzymes and biochemicals were obtained from Boehringer Mannheim and Sigma. Tissue culture reagents and media were purchased from Life Technologies, Inc.

Cell Cultures and Virus—VSV New Jersey serotype, Ogden strain was purified as described previously (4, 22) from baby hamster kidney cells (BHK-21, ATCC CCL 10) by incubating with virus at a multiplicity of infection of 0.05. BHK cells were maintained in Eagle's minimal essential media supplemented with 7% fetal bovine serum.

Purification of Recombinant P Proteins from E. coli—Various recombinant P mutant proteins were expressed in E. coli and purified essentially as described previously (4). After purification by phosphocellulose and DE52 column chromatography, the P proteins were more than 90% pure.

CD Spectroscopy of P Protein—CD spectra of various P mutant proteins were measured at 25°C on a Jasco J-600 spectropolarimeter interfaced to a computer for data collection and manipulation. The instrument was calibrated with d-10 camphorsulfonic acid (25). Spectra were recorded between 260 and 190 nm using a 0.01-mm quartz cell. All spectra, averages of at least 10 scans, are correlated for the spectral contributions of the buffer. The measurements were performed at varying protein concentrations in a solution containing 20 mM Hepes (pH 7.5), 50 mM NaCl. The protein concentration was determined according to Beaven and Holiday (28). The mean residue mass was calculated from the amino acid composition (26). The spectra were analyzed to estimate the distribution of secondary structural elements by the variable selection method developed by Manavalan and Janson (27).

Gel Filtration Analysis of P Protein—Bacterially expressed, purified P protein at various concentrations was chromatographed through a Sephadex G-100 gel filtration column. An 18-ml bed volume column was first equilibrated with VSV transcription buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, 2 mM DTT) and subsequently calibrated with standard proteins of known molecular size. We used bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) (purchased from Sigma) as known protein standards. P protein (5 μg) in a 100-μl VSV transcription buffer was fractionated, and the alternate fractions were monitored by SDS-PAGE, followed by silver staining and autoradiography where applicable. For unphosphorylated and P59/61 mutant P proteins, the fractions were analyzed either by silver stain or by autoradiography of ³⁵S-labeled proteins. On the other hand, for phosphorylated P proteins, bacterially expressed P0 and P41+61 mutant proteins were first phosphorylated with recombinant CKII and [γ-³²P]ATP in vitro in a VSV transcription buffer and chromatographed through Sephadex G-100 in the same way as described above. The P protein band in the SDS gel was identified by its characteristic mobility (molecular mass ~46 kDa) as well as by Western blot analyses with specific antibody.

Protein Kinase Assay—In the standard protein kinase assay, a varying amount (0.1-1 μg) of the desired form of P protein (P0, P1, or mutant P proteins) was incubated in the presence of CKII (0.01 milli- uM), 10 mg purified VSV was initially diluted in a buffer containing 0.4 M NaCl, 10 mM Tris-HCl (pH 8.0), 5% glycerol, 2% Triton X-100, 1 mM DTT, by incubation on ice for 90 min with continuous gentle rocking. The viral ribonucleoprotein (i.e., the genomic RNA associated with N, P, and L proteins) was then purified by centrifugation onto a 100% glycerol cushion through 30%glycerol containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 2 mM MgCl₂, 1 mM DTT for 2 h at 45 K in an SW50.1 rotor at 4°C. Ribonucleoprotein was collected from the top of a 100% glycerol cushion in 2 ml of Tris-EDTA and was disrupted again as described above, but in buffer containing 1 M NaCl and 0.5% Triton X-100 to dissociate the L and the P proteins from the N-RNA template. The N-RNA was purified by centrifugation through 30% glycerol onto a 100% glycerol cushion in the same way as described above. The salt supernatant containing L and P proteins was stored at ~80°C until further use. N-RNA was further purified by an additional high salt wash, centrifuged through 15% Renografin onto a 76% Renografin cushion (23), followed by two serial recoveries from CsCl gradients (0.35 mg of CsCl/ml of Tris-EDTA final concentration) by centrifugation at 45 K for 16 h in an SW60 rotor at 20°C. N-RNA was finally dialyzed against Tris-EDTA. The purity of the N-RNA template was determined initially by silver staining of gels after SDS-PAGE and finally by reconstitution of transcription in vitro with recombinant (29) or viral L protein and bacterially expressed P protein (4).

The high salt fraction containing L and P was dialyzed against phosphocellulose buffer (20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM DTT) and loaded onto a 2.0-ml phosphocellulose column equilibrated with the same buffer. The column was washed with phosphocellulose buffer, and the bound L protein was eluted with a 0–1.0 M NaCl gradient (12 ml) in the same buffer. Fractions in which L was completely free of P protein (as identified by silver staining) and devoid of cellular kinase (as checked by phosphorylation of bacterially expressed VSV P protein as substrate) were pooled. If necessary, the L protein was further rechromatographed on a second phosphocellulose column to remove any contaminating viral P protein and cellular kinase.

Reconstitution of VSV Transcription in Vitro—VSV transcription in vitro was carried out essentially as described earlier (23) except that E. coli, recombinant P proteins were used instead of viral P protein. 15 ng of N-RNA, approximately 50 ng of L, and 50 ng of P protein were included in a 25-μl reaction mixture which also contained VSV transcription buffer, 0.5 mM each of ATP, CTP, GTP, 100 μM UTP, 10 μCi of [α-³²P]UTP and 1 unit/ml RNasin. Reactions were incubated for 2 h at 30°C, and poly(A) tails were removed from the viral messages by treating with 100 ng of oligo(dT) and 1 unit of RNase H at 37°C for 15 min. Reactions were terminated by extraction with phenol-chloroform and precipitated with ethanol in the presence of 5 μg of carrier RNA. Viral RNA products were analyzed by electrophoresis on 5% polyacrylamide gel containing 7 M urea as described earlier (23).

Other Procedures—Quantitative silver staining of polyacylamide gels was done by using Bio-Rad reagents and protocols. Radioactivity of labeled protein fractions was measured in a model LS 1701 Beckman liquid scintillation counter. Where needed, densitometric scanning of stained protein bands or autoradiograms were performed in a model 620 Bio-Rad video densitometer.

**RESULTS**

CD Spectroscopy of P Protein—The cascade phosphorylation pathway proposed by Barik and Banerjee (5) strongly suggests that the phosphorylation of P0 to P1 must initiate a conformational change in the protein which presumably imparts its transactivation property. Since the prediction of the secondary structure for globular proteins from circular dichroism spectroscopic data is a well established method in recent years (27), we were interested in comparing the secondary structures of P0
and P1 proteins using far UV circular dichroism spectroscopy. As shown in Fig. 1 and Table I, analysis of P0 predicts a secondary structure distribution of 27% \(\alpha\)-helix and 23% \(\beta\)-structure. Interestingly, a dramatic change from \(\beta\)-structure to \(\alpha\)-helical structure is predicted when the P protein is phosphorylated by CKII. The predicted secondary structure of P1 consists of 48% \(\alpha\)-helix and only 7% \(\beta\)-structure (Table I), i.e. an increase of approximately 2-fold in helical content over unphosphorylated P0. We have shown previously that Ser-59 and Ser-61 residues of the P protein are phosphorylated by CKII, and when both serine residues are altered to alanine (Fig. 3, P59/61), the mutant P protein became inactive in transcription (18). When the structure of the P mutant was analyzed by CD, the mutant protein showed similar overall secondary structure as that of P0 protein (Fig. 1, Table I). These results strongly suggest that there is a significant difference in the secondary structures between P0 and P1 proteins. The conformation of P1 is more \(\alpha\)-helical in nature similar to many well studied eucaryotic transcription factors.

CKII-mediated phosphorylation of Domain I facilitates the apparent dimerization of P protein. Since the activities of many transcription factors have also been shown to depend on their ability to form homodimers (24), we were interested to examine whether phosphorylated P protein (P1) can oligomerize in vitro. To do so, we expressed various P mutants in bacteria and used the purified proteins for such studies. The P0 at a concentration of 50 \(\mu\)g/ml was subjected to chromatography in Sephadex G-100 gel filtration column along with standard proteins of known molecular size as detailed under “Experimental Procedures.” Fractions were analyzed by SDS-PAGE, and the P protein band was identified by its characteristic mobility (M, \(-46,000\)) after silver staining or autoradiography (in the case of \(\gamma\)-labeled protein). A densitometric scan of a representative fraction (Fig. 2, top panel) shows that essentially all of the P protein eluted at a position consistent with a 29-kDa monomeric polypeptide, suggesting that in the unphosphorylated form, P protein exists as a monomer at 50 \(\mu\)g/ml concentration. However, at a high protein concentration, i.e. 200 \(\mu\)g/ml, P0 eluted at a position higher than the 66-kDa marker, indicating that the unphosphorylated P protein has a natural propensity to form oligomer at high concentration (data not shown).

Next, the P0 protein was phosphorylated by CKII to form P1 in the presence of [\(\gamma\)-32P]ATP in vitro and tested its size estimated in the same manner as described above. Interestingly, P1 eluted at a position consistent with its being a dimer at the same protein concentration, i.e. 50 \(\mu\)g/ml, at which P0 fractionated as a monomer (Fig. 2, middle panel). A small amount of remaining unphosphorylated P0 was fractionated as a monomer (Fig. 2, lower panel). These results strongly suggest that CKII-mediated phosphorylation brings about changes in the secondary structure of the P protein and perhaps facilitates apparent dimerization of P monomer.

Role of Single Phosphorylation in P Function—We were especially interested in two P mutants (Fig. 3), e.g. P4+59 (where Ser-59 is unaltered but four other possible phosphorylation sites are mutated to alanine) or P4+61 (where Ser-61 is unaltered and four other sites are mutated to alanine), which when expressed in COS cells, were phosphorylated to the same extent as that of wild-type P protein (18). However, these mutants were transcriptionally inactive in vitro, when transcription-reconstitution was performed using wheat germ-translated proteins (18). To resolve the discrepancy between the in vivo and in vitro data, we expressed these single serine mutant P proteins in E. coli and examined their elution profile in Sephadex G-100 column chromatography as described above. Unexpectedly, when these mutant proteins were phosphorylated by CKII in vitro and subjected to gel filtration analysis, they eluted in the same manner as that of wild-type P1 (Fig. 4, top panel), suggesting that they form apparent dimers at low protein concentration (50 \(\mu\)g/ml). We reasoned that the inability of P4+61 and P4+59 to transactivate L protein as observed before may be due to incomplete phosphorylation of the single serine residues by CKII present in wheat germ extract during their translation in vitro due possibly to their low affinity for CKII (18). Whereas, when these mutant proteins were completely phosphorylated by recombinant CKII in vitro, they were able to form dimers, thus, transcriptionally active.

To test this possibility, we used bacterially expressed P mutant proteins and repeated our previous experiments. Purified CsCl-banded N-RNA template and kinase-free L protein from purified virions were prepared as described under “Experimental Procedures” and used in transcription reconstitution reaction using bacterially expressed wild-type as well as P4+59 and P4+61 mutant proteins. As shown in Fig. 5, both P4+61 and P4+59 mutant proteins were unable to support viral transcription in the presence of N-RNA and L protein. The P0 (denoted Pwt, Fig. 5), on the other hand, showed the expected transcriptional activity under the same experimental conditions. As noted earlier, the trace quantity of CKII present in the purified N-RNA template efficiently activated P0 (Pwt) but failed to do so for the single serine mutants. However, when similar transcription reactions were performed in the presence of excess recombinant CKII, both the mutant proteins were transcriptionally active almost to the same extent as P0 (Pwt) (Fig. 6). These results indicate that P4+61 and P4+59 potentially need increased concentrations of CKII for their activation. Mutation in a single Ser residue has increased its requirement for CKII for phosphorylation leading to activation. To confirm whether CKII present in the N-RNA template was indeed unable to phosphorylate P4+61 and P4+59, we performed an in vitro phosphorylation reaction using the same amount of N-RNA template (0.5 \(\mu\)g in 25 \(\mu\)l) as the source of CKII and 100 ng each of the P proteins as substrate in the transcription reaction. The results shown in Fig. 7 indicate that...
the amount of CKII present in the N-RNA template was indeed unable to phosphorylate P4\textsuperscript{161} and P4\textsuperscript{159}. In contrast, when excess recombinant CKII was added to the reaction mixture, both mutant proteins were phosphorylated completely. These results strongly suggest that P4\textsuperscript{159} and P4\textsuperscript{161} have lower affinity for CKII, but once phosphorylated by excess CKII can support VSV transcription. Thus, we conclude that phospho-

**TABLE I**

CD analyses of P proteins

CD spectroscopy of P0 (unphosphorylated), P1 (phosphorylated by CKII), and P59/61 were carried out in VSV transcription buffer. The secondary structures were estimated from the spectra by using variable selection method. Secondary structures: H, \(\alpha\)-helix; AB, antiparallel \(\beta\)-sheet; PB, parallel \(\beta\)-sheet; T, \(\beta\)-turn; O, other structures; TOT, total.

| P proteins | Calculated secondary structural percentages |
|------------|--------------------------------------------|
|            | H  | AB | PB | T  | O  | TOT |
| P0         | 27 | 21 | 02 | 26 | 27 | 103 |
| P1         | 48 | 05 | 02 | 21 | 26 | 102 |
| P59/61     | 32 | 13 | 03 | 37 | 16 | 101 |

**Fig. 2. Elution profile of the P protein from gel filtration column.** Wild-type P protein was fractionated through a Sephadex G-100 column as described under "Experimental Procedures." Positions of the two standard markers (66k and 29k) are shown on the top. Top panel, fractionation of unphosphorylated P protein. P0 peak was monitored either by silver staining or by labeling the protein with \(^{[35}S\) methionine (\(\bullet\)). Middle panel, fractionation of a mixture of unphosphorylated (P0) and phosphorylated P1 protein. The P1 peak was monitored by silver staining (\(\bullet\)) as well as \(^{32}P\) counting (\(\bigcirc\)), arbitrary scale not shown. Bottom panel, the fractionation of P59/61 (phosphorylation defective mutant).

**Fig. 3. Schematic representation of VSV(NJ) P protein.** The entire P protein containing all the domains is shown. The solid dots in domain I and II represent phosphorylation sites in these regions. An enlargement of a part of domain I shows the locations of serine residues 59 and 61 that are phosphorylated by CKII. The different mutant P proteins are also shown. In the case of P59/61, both Ser-59 and Ser-61 are mutated as indicated by asterisks, whereas in the case of single mutants, either Ser-59 or Ser-61 is unaltered, but four other possible sites are mutated (18).

**Fig. 4. Elution profile of single serine mutant P protein from gel filtration column.** The P mutant protein was fractionated through Sephadex G-100 column as described in the legend to Fig. 2. The top panel represents the fractionation of P4+61 as identified by silver staining (\(\bullet\)) and \(^{32}P\) counting (\(\bigcirc\)), whereas the bottom panel shows the result for P0, for comparison.

**Fig. 5. Transcription of N-RNA template by P proteins.** In vitro transcriptions were reconstituted using CsCl-banded N-RNA template and cellular kinase free L protein along with various bacterially expressed mutant P proteins as described in the text. The corresponding viral mRNAs are indicated by G, N, P, and M. + indicates presence and − indicates absence. Note that Pwt signifies the bacterially expressed unphosphorylated P0 form.
rylation of either Ser-59 or Ser-61 is necessary and sufficient to transactivate L-polymerase, although alteration of one serine residue significantly decreases its affinity for CKII. To further demonstrate that complete phosphorylation of P4

1

59 or P4

1

61 by CKII brings about similar conformational change as observed for P1, we carried out CD analyses of the mutant proteins. As shown in Fig. 8 and Table II, phosphorylated P4

1

61, as expected, exhibited similar increases in α-helical structure as the wild-type P1.

Phosphorylation of P Mutants by L-kinase—Next, we were interested to study whether CKII-mediated phosphorylation is a prerequisite to the subsequent phosphorylation by L-associated kinase (5). To do so, we performed standard protein kinase assay by using CKII-free L protein purified from viral ribonucleoprotein complex as described under “Experimental Procedures.” As shown in Fig. 9, the L protein failed to phosphorylate P0, confirming that P0 is not the substrate for L-kinase (5). Similarly, L-kinase was unable to phosphorylate the P mutant where both Ser-59 and Ser-61 were altered. In contrast, when P1 was used as the substrate in the L-kinase reaction, L protein could effectively phosphorylate P1 to produce P2 as observed earlier (5). As expected, the phosphorylated single P mutants (either P4

1

61 or P4

1

59), which are transcriptionally active, were further phosphorylated by L-kinase. These results indicate that the P mutants, which can attain the similar structure as that of P1 after phosphorylation by CKII, are the proper substrates for L-kinase, suggesting that L protein recognizes only the phosphorylated form of the P protein.

Role of CKII-mediated Phosphorylation on the Template Binding Activity of the P Protein—Since the physical and functional interactions between the N-RNA template and the P protein are required for the RNA synthetic process, we wanted to study whether the phosphorylation by CKII facilitates binding of the P protein to the N-RNA template in vitro. We used purified 35S-labeled unphosphorylated (P0 and P59/61) and 32P-labeled phosphorylated P proteins (P1 and single serine mutants) for this assay. N-RNA template (2 m), was incubated with increasing amounts of each of the P mutant proteins in a reaction volume of 50 ml, and binding of P protein to the template was quantified by the densitometric scanning of the autoradiogram as detailed under “Experimental Procedures.” The results shown in Fig. 10 indicate that P0 and P59/61 bind to the template in a manner which is sigmoidal in nature (K0.5 approximately 2 \times 10^{-6} M), whereas P1, P4+61, or P4+59 (data not shown) bound in a linear fashion, indicating stoichiometric binding (K0.5 10^{-6} M). This suggests that both P1 and phosphorylated single P mutant proteins interact with the N-RNA template with higher affinity than the unphosphorylated P protein. Thus, it seems that change in conformation and the apparent dimerization induced by CKII facilitate the template binding activity.

Inhibition of Viral Transcription by Double Mutant P Protein—Since the P mutant P59/61 is transcriptionally inactive due to its inability to be phosphorylated by CKII, it was of interest to examine whether this mutant protein can act as a transdominant negative mutant. To test the above possibility, we carried out in vitro transcription reconstitution assay by using increasing amounts of P59/61 mutant protein to study its effect, if any, on transcriptional activity in the presence of P0 protein, which is phosphorylated by CKII associated with the N-RNA template. The results shown in Fig. 11 demonstrate...
that only 2-fold molar excess of P59/61 mutant protein completely inhibited the transcriptional activity of wild-type P protein. However, addition of 10-fold molar excess of wild-type P protein in the same reaction did not show any inhibitory effect (data not shown), indicating that P59/61 mutant indeed acts as a transdominant negative inhibitor.

DISCUSSION

It is becoming increasingly apparent that cellular protein kinases play important roles in the life cycle of several nonsegmented negative strand RNA viruses (33–35). It appears that the virus structural protein P, which is a transcription factor for such groups of viruses, needs to be phosphorylated by a specific cellular protein kinase for transactivation of the RNA-dependent RNA polymerase (L). Recent work from our laboratory indicates that a cascade phosphorylation is operative for the activation of the P protein of VSV (4–6). First, the cellular CKII phosphorylates two serine residues 59 and 61 within the acidic domain I of unphosphorylated P0 rendering it biologically active (P1 form) (18). The P1 form is then phosphorylated within the C-terminal domain II to P2 form by L-associated protein kinase during transcription in vitro and presumably in vivo. However, the exact role played by the phosphorylated serine residues 59 and 61 to activate P protein and imparting the transactivation property remains unclear. It is conceivable that phosphorylation of serine residues brings about a change in the secondary structure of the P protein that facilitates its binding to the L protein as well as the N-RNA template.

To probe into this possible structural alteration of the P protein, we performed CD analyses of various P mutant proteins, and the results obtained from such analyses strongly support the contention that the phosphorylation by CKII indeed imparts a significant effect in the secondary structure of the P protein. The phosphorylated P1 form is predicted to have a high degree of \( \alpha \)-helical structure (48%) compared with unphosphorylated P0 which is predicted to contain only 27% \( \alpha \)-helix, similar to that of phosphorylation defective double mutant P59/61 (Fig. 1, Table I). Presumably, the increased \( \alpha \)-helical structure directly plays an important role for the transactivation property of the P protein. Consistent with this observation, many eucaryotic transcription factors have also been shown to possess extensive \( \alpha \)-helical structures that are implicated in binding to cognate proteins or promoter se-

**TABLE II**

CD spectroscopy of P4+61 was carried out as described in Table I. The estimated secondary structures of P4+61 were compared with that of P1.

| P proteins | H | AB | PB | T | O | TOT |
|------------|---|----|----|---|---|-----|
| P4+61      | 45 | 12 | 09 | 33| 05| 104 |
| P1         | 48 | 05 | 02 | 21| 26| 102 |

FIG. 9. Phosphorylation of P proteins by L-associated kinase. The unphosphorylated (P0) and phosphorylated (P1) forms of indicated P proteins were incubated with highly purified L protein (~1 \( \mu \)g) in a standard protein kinase reaction as described under "Experimental Procedures." In case of P0, 1 \( \mu \)g of P protein was used in a 20-\( \mu \)l kinase reaction. However, in the case of P1, 5 \( \mu \)g of bacterially expressed unphosphorylated P protein was first phosphorylated with 0.05 milli-units of CKII in a 100-\( \mu \)l reaction mixture containing 0.5 mM ATP. The unlabeled P1 was then purified by DE52 column chromatography. Approximately 1.0 \( \mu \)g of P1 protein was used in kinase reaction by L. + indicates presence and − indicates absence of corresponding agent.

FIG. 10. Binding of P proteins to the N-RNA template. Indicated amount of P protein was incubated with 2 \( \mu \)g of N-RNA template in a 50-\( \mu \)l reaction mixture containing VSV transcription buffer for 1 h at 30 °C as described under "Experimental Procedures." The amount of P protein bound to the template was determined from the autoradiogram of the gel using a Bio-Rad densitometer scanner, and the relative value was plotted against the amount of P protein added. We used \( ^{35} \)S-labeled protein in the case of P0 and P59/61, whereas \( ^{32} \)P-labeled protein was used in the case of P1 and P4+61. \( \square \), unphosphorylated P0; \( \bullet \), P59/61 mutant; ○, P1; ●, P4+61 mutant.

FIG. 11. Inhibitory effect of P59/61 in VSV transcription. An indicated amount of P59/61 mutant protein was added to the transcription reaction containing a constant amount of N-RNA template (500 ng) and wild-type P protein (100 ng). The transcription reactions were processed as described under "Experimental Procedures." Solid line indicates transcription with wild-type P only that is considered as 100% transcription. ○, indicates reaction where both wild-type and mutant P proteins were included.
quences on DNA (30). Moreover, three-dimensional structural analyses have shown that phosphorylation can affect protein activity by inducing allosteric conformational changes, as well as by electrostatic repulsive effects, and these mechanisms are both likely to be important in regulating the function of transcription factors (20, 21). This alteration of the secondary structure possibly induces efficient dimerization of the transcription factors for their functional activity. Similar phenomena seem to be operative for the P protein as it relates to its function. Based on the results obtained from gel filtration analyses, it seems that the wild-type P protein, in its unphosphorylated form, undergoes dimerization according to the reversible reaction: 

$$2(P0) \rightarrow (P1)2$$

Phosphorylation of the P protein at Ser-59 and Ser-61 facilitates the dimerization process by increasing the association constant of the monomers such that the reaction 2(P1) → (P1)2 takes place at a low protein concentration. Alternatively, since gel filtration measures a change in the Stokes radius of the protein, it is possible that phosphorylation of the P0 protein simply changes its shape and not its state of oligomerization. A direct measurement like chemical cross-linking or sedimentation analysis need to be performed to come to a definite conclusion. Thus, it seems that the first step toward activation of P protein by CKII is most likely the alteration of the secondary structure with apparent dimerization of the protein. The P protein then binds with the L protein and the N-RNA template to initiate the RNA synthetic process. How precisely the latter process manifests still remains an enigma. While this work was in the review process, Gao and Lenard (39) have reported that the active P1 protein of VSV (Indiana serotype) exists as multimeric, probably tetrameric, structure as determined by gel filtration and cross-linking analyses. Whether this apparent discrepancy relates to the different serotypes of the P protein used in these studies remains to be determined.

Using two single serine mutants, i.e. mutant P4+61 or P4+59, we have shown that these mutants have low affinity for CKII for phosphorylation such that at low concentration of CKII, these mutants are poorly phosphorylated and accordingly transcriptionally ineffective. However, in the presence of a high concentration of CKII, the single mutants are not only completely phosphorylated but also dimerized apparently and transcriptionally active as the wild-type P1. Therefore, a single serine phosphorylation is necessary and sufficient to activate the P protein. Thus, two serine residues are strategically located within the acidic domain of the P protein in such a way that phosphorylation by a low amount of CKII allows it to rapidly fold into its proper structure. It seems that the proper structural alteration of the P protein is a prerequisite for its activation. In this respect, it would be interesting to find out whether the chimeric protein in which the acidic domain I has been replaced by apparently unrelated acidic polypeptide, such as tubulin, possesses a structure similar to that of P1. Earlier studies have shown that such chimeric P protein was transcriptionally active in an in vitro transcription reconstitution reaction (32).

Our results further demonstrate that subsequent phosphorylation of the P protein by L-associated kinase and its efficient binding to the N-RNA template are all dependent on prior phosphorylation by CKII. In this connection, it is interesting to note that genetic complementation data and the presence of excess P protein over L protein in the viral transcription complex have led to the suggestion that the functional VSV polymerase may consists of one polypeptide of L and two of P, i.e.

$$L(P)_2$$

It is interesting to note that the phosphorylation-defective P mutant (P59/61) is transcriptionally inactive due to its inability to undergo the phosphorylation process that controls the proper folded structure of the P protein. Consistent with this result, the CD analysis also suggests that the secondary structure of this double mutant is identical to that of unphosphorylated P protein (P0). However, this double mutant acts as a transdominant negative mutant in an in vitro transcription reconstitution with wild-type P protein (Fig. 11). It remains to be seen whether this mutant forms a heterodimer with wild-type P protein that leads to the formation of an inactive L(P-P59/61)2 complex. Regardless of the mechanism of inhibition of viral transcription by this double mutant, this mutant may be utilized for generating a resistant cell line to VSV.

Finally, it seems that the P protein behaves as an RNA virus transcription factor where phosphorylation causes a major structural change which mediates efficient binding of the P protein to the N-RNA template and presumably L protein. In this respect, the P protein behaves like several eucaryotic transcription factors where phosphorylation promotes dimerization and binding to the DNA template (19, 24). Detailed structural studies of the P protein, e.g. crystallography, NMR studies, etc., would certainly aid us understanding its three-dimensional structure as it relates to its function. The knowledge derived from the above studies would certainly provide the opportunity to understand whether the phosphorylation pathway as demonstrated in VSV is unique to other viruses like rabies, measles, mumps, respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), etc., which employ the same strategy as VSV to invade cells. Recently, the cellular protein kinases that phosphorylate the P proteins of several RNA viruses have been identified. Similar to VSV, both RSV P (35, 36) and measles P (37) proteins have been shown to be phosphorylated by the same cellular enzyme, CKII. In contrast, the cellular protein kinase that phosphorylates HPIV-3 P protein was found to be indistinguishable from cellular protein kinase C subtype ζ (38). It is noteworthy that in all the cases, phosphorylation by either CKII or PKC takes place within the acidic domains of the P proteins in spite of the difference in their relative sites within the polypeptide. Thus, it will be interesting to see whether the introduction of phosphate groups brings about similar conformational changes (as found in VSV P) in the acidic domains of measles, RSV, or HPIV-3 P proteins, which in turn lead to their transcriptional activation. Involvement of different protein kinases in the regulation of gene expression of these RNA viruses suggests that these viruses might infect the host organ in a tissue-specific manner where the required source of the essential protein kinase is available. Further studies along these lines would certainly be important to design and develop antiviral agents specifically directed to the cellular kinases which have an intimate relationship with the virus’s life cycle.

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