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Single Serine Phosphorylation within the Acidic Domain of Chandipura Virus P Protein Regulates the Transcription *in Vitro*

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Bacterially expressed unphosphorylated P protein of Chandipura Virus was found to be efficiently phosphorylated *in vitro* by casein kinase II (CKII). The phosphorylated form of the P protein supported the transcription *in vitro* but the unphosphorylated form could not. Kinetic data suggests that CKII incorporates one molecule of phosphate. Western blotting with monoclonal antibody against phosphoserine and phosphoaminoacid analysis confirmed that the phosphate accepting residue was serine. Comparison with P protein of other viruses and tryptic digest of the phosphorylated protein predicted the ser^{62} was the probable site for phosphorylation. This was further confirmed by substituting ser^{62} with alanine by site-directed mutagenesis. CKII was unable to phosphorylate the mutated P protein which in turn could not support the transcription *in vitro*. The phosphorylated P protein eluted from the gel filtration at the position of its dimer in contrast to the unphosphorylated protein which eluted as monomer.

INTRODUCTION

Chandipura (CHP) virus, a member of the *rhabdoviridae* family was first isolated from a human female from a village of the same name in India (Bhatt and Rodrigues, 1967). Though this virus is not studied in detail, it appears from the analysis of transcription initiation sequences and from the complementation studies that the organization of its genome is very similar to that of the VSV, the prototype rhabdovirus (Chang *et al.,* 1974; Gadkari and Pringle, 1980a,b). The Rhabdoviruses have a nonsegmented linear, negative sense RNA genome which is wrapped with nucleocapsid protein N. In VSV, it has been well documented that the RNA genome is transcribed and replicated by RNA-dependent RNA polymerase packaged with the virion. The RNA-dependent RNA polymerase is composed of two polypeptides, the large protein L (241 kDa) which resides most of the catalytic activity required to carry on both of the processes and a phosphoprotein P (~30 kDa).

Over the past several years the role of phosphoprotein P in transcription and replication is being studied in detail by several groups. The protein is present in a variety of phosphorylated forms inside the virion (Clinton *et al.,* 1979; Hsu and Kingsbury, 1982). The role of phosphorylation in the activity of P protein is currently being resolved by expressing the gene in bacteria in an unphosphorylated form. Using the P protein from the VSV New Jersey, Barik and Banerjee (1992a) have demonstrated that the protein exists in three different forms, the unphosphorylated form P0, which is phosphorylated by the host kinase to give P1, which is phosphorylated further by L-associated kinase to give hyperphosphorylated form P2. The host kinase was purified from uninfected cell extract and found indistinguishable from Casein Kinase II (1992b). CKII is packaged within the virion and involved in P protein phosphorylation also *in vivo* (Gupta *et al.,* 1995; Chen *et al.,* 1997). In the *in vitro* reconstitution assays, CKII-mediated phosphorylation of the protein was found essential for the transcription of the virus (Barik and Banerjee, 1992b). The specific phosphoacceptor sites have been mapped. In the case of VSV(NJ) the phosphorylation-negative mutant S59A/S61A of P protein was found completely inactive in *in vitro* transcription (Takacs *et al.,* 1992). The phosphorylation also appears to be involved in the possible dimerization and change in the alpha-helical content of the protein (Das *et al.,* 1995), but it has no role in the N-P interaction (Takacs *et al.,* 1993). Sites for the L-associated kinase were also mapped. In P of VSV(NJ) the double mutant S236A/S242A was found inactive in *in vitro* transcription (Chattopadhyay and Banerjee, 1987).

The essentiality of P protein phosphorylation in VSV Indiana transcription is still a puzzle. Both the CKII and L-associated kinases are involved in the phosphorylation of P-IND, but unlike that of VSV(NJ), the phosphorylation negative double mutant (S60A/S64A) of P protein behaved like wild type when expressed in bacteria. When expressed in COS cells, the same mutant showed only about 10–15% activity (Mathur *et al.,* in press). Spadafora *et al.* (1997) have reported that the same mutant is about 30% active when used in higher concentration. Unlike P-NJ, Gao and Lenard (1996) found no alteration in the CD spectra of the bacterially expressed P-IND protein before

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and after CKII-mediated phosphorylation. The essentiality of the L-associated kinase in P protein activity is also called into question (Gao and Lenard, 1995a). Using highly purified L protein devoid of any detectable kinase activity, Gao and Lenard showed that CKII-mediated phosphorylation is sufficient for P-IND activity. Phosphorylated P protein has been shown to form homotrimer unlike P-NJ and this facilitates the binding of the protein to L- and N-RNA complex (Gao and Lenard, 1995b). But as in VSV-NJ, phosphorylation of P-IND has no role in N-P interaction (Gupta and Banerjee, 1997).

In an attempt to address the above questions in the life cycle of another virus in the same group we have chosen CHP virus, a human pathogen, and an evolutionarily distant virus to VSV. From the sequence analysis of the genome it appears that it is equally distant from each of the two serotypes of VSV. Among the genes so far sequenced, the P gene is mostly diverged, having only about 20% homology to the same protein in VSV. To compare the similar studies in CHP P protein, a full-length clone of CHP virus P gene was constructed and expressed in bacteria. The host-derived kinase involved in the phosphorylation of CHP virus P protein was identified to be casein kinase II (Chattopadhyay and Chattopadhyay, 1994). In this communication, to understand the role of CKII-mediated phosphorylation in controlling the biological activity of P protein, the bacterially expressed protein was used in the transcription reaction, reconstituted for the first time in the case of CHP virus. Phosphoamino acid and phosphopeptide analysis demonstrated that phosphorylation of P protein by CKII was exclusively at serine residue that map in the amino-terminal domain I region. In contrast to the VSV P proteins, in CHP virus P protein a single serine phosphorylation was found to be sufficient for supporting the transcriptional activation. The phosphoacceptor serine was identified by mutagenesis. Replacement of that residue with alanine abolished the biological activity of the protein. Like P-NJ, the CHP virus P protein was eluted at the position of its dimer in the gel filtration.

MATERIALS AND METHODS

Cell culture and virus

CHP virus (Strain 1653514) was purified as described previously (Masters and Banerjee, 1986) from Baby Hamster Kidney (BHK-21) cells by infection with virus at a multiplicity of infection of 0.1. BHK-21 cells were grown in Glasgow modified minimal essential medium supplemented with 5% New born calf serum.

Purification of CHP virus transcription components

Ten milligrams of purified virus was disrupted with low salt lysis buffer containing 1.85% Triton X-100 in 10 mM Tris–HCl, pH 8.0, 5% (v/v) glycerol, 0.4 M NaCl, 0.6 mM DTT at 4°C for 60 min. The viral nucleoprotein was sedimented by centrifugation in a SW60 rotor at 40,000 for 2 h through 30% glycerol in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA on to a 100% glycerol cushion. The cushion was diluted with 2 ml Tris-EDTA. The viral RNP was again disrupted with high salt lysis buffer containing 0.8 M NaCl in 10 mM Tris–HCl, pH 8.0, 5% (v/v) glycerol, 0.5% Triton X-100, 0.6 mM DTT as above. The N-RNA was sedimented by centrifugation in an SW60 rotor at 45,000 rpm for 2 h through 30% glycerol on to a 100% glycerol cushion. The released L and P proteins on the top of the glycerol gradient was collected for further purification. To purify the N-RNA, cushion was diluted with 2 ml Tris–EDTA and mixed with 0.2 ml high salt lysis buffer and incubated at 4°C for 1 h. The mixture was centrifuged through 15% Renographin solution in 10 mM Tris–HCl, pH 8.0, 10 mM KCl, 15 mM $MgCl_2$ at 45,000 rpm for 4 h on a 76% Renographin cushion. Further purification was done by two serial recoveries of N-RNA band from CsCl gradient (0.35 g/ml in Tris–EDTA final concentration) by centrifugation at 45,000 rpm for 16 h in SW60 rotor at 20°C and then dialyzing against 10 mM Tris–HCl, pH 8.0, 50 mM NaCl.

L and P proteins released from the viral RNA with high salt lysis buffer were recovered from the 30% glycerol and dialyzed against 20 mM Tris–HCl, pH 7.4, 10% glycerol, 0.1% Triton X-100, 0.3 mM DTT and chromatographed through a 2-ml phosphocellulose column preequilibrated with the same buffer. The unbound fraction composed of P protein was concentrated with Amicon concentrator. The column was washed with 2× column volume of equilbration buffer containing 0.5 M NaCl and L protein was eluted with equilibration buffer containing 1 M NaCl. The L protein containing fractions were concentrated and rechromatographed in the same column for further purification.

Purification of recombinant P protein from bacteria

P gene of CHP virus cloned in pET3a vector was expressed in *Escherichia coli* BL21DE3 and extracted from the inclusion bodies as described earlier. The protein was loaded in Q-Sepharose column equilibrated with 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1% Triton X-100 and eluted with a 0–500 mM NaCl gradient.

Quantitation of proteins

Protein concentration was quantitated by the absorbance at 280 nm and calculated according to the following equation. Molar exinction coefficient was calculated from the sequence of the protein.

$E = 20{,}000\ M^{-1}cm^{-1}$ (molar extinction coefficient)

$A_{280} = Ecl$

$c = A/El$ M,

where $l = 1$ cm (path length) and c = concentration (molar).

In vitro phosphorylation

In the standard protein kinase assay, varied amounts of (1–5 μg) purified bacterially expressed protein was incubated in the presence of 0.01 milliunit of recombinant human CKII (Boehringer-Mannheim) or 5 μg BHK cell extract in a transcription buffer containing 10 μCi [γ-^{32}P]ATP for 1 h at 30°C. According to need, the reaction mixture was used directly or stopped either with lysis buffer or spotted on Whatman P81 paper and washed with 5% TCA containing 75 mM phosphate buffer and radioactive count was measured in the scintillation counter.

Reconstitution of CHP virus transcription in vitro

CHP virus transcription *in vitro* was carried out essentially as described earlier in the case of VSV (De and Banerjee, 1984), except that *E. coli*-expressed, recombinant P proteins were used instead of viral P protein where mentioned. Approximately 750 ng of N-RNA, 100 ng of L protein, and 200ng of bacterially expressed P protein or 100ng of viral P protein (saturating amount) were used in a 25-μl reaction mixture containing 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM DTT, 50 μM UTP, 1 mM each of ATP, GTP, CTP, 10 μCi [α-^{32}P]UTP, and 1 unit RNasin. The reaction mixture was incubated at 30°C for 2 h and then chased with 1 mM of UTP for 10 min. Poly(A) tails were removed form the transcripts by incubating the reaction mixture with 100 ng oligo $(dT)_{18}$ and 0.5 U of RNase H for another 10 min. The reaction mixture was either spotted on DE81 paper and washed with 0.5 M Na-phosphate buffer, pH 7.4, and radioactive count was measured or extracted with phenol:chloroform isoamylalcohol and precipitated with ethanol, electrophoresed in 5% polyacrylamide–7 M urea gel. Then 0.01 mU of recombinant CKII was added to the reaction mixture for the P protein phosphorylation where mentioned.

Peptide mapping of phosphorylated P protein

In vitro ^{32}P-labeled P proteins were resolved in a Tricine–SDS–polyacrylamide gel (16.5% T, 3% C), according to the procedure of Schagger and Jagow (1987). Half of the digestion reaction aliquoted after 2 h of incubation was stopped with TLCK (tosyllysine chloromethyl ketone, a trypsin inhibitor) at a concentration of 50 μg/ml. The partial and totally digested samples were boiled with SDS sample buffer and electrophoresed accordingly. The dried gel was autoradiographed.

Phospho amino acid analysis

P protein was phosphorylated *in vitro* using [γ-^{32}P]ATP. Free nucleotides were separated by Sephadex G-25 spun column. The phosphorylated protein was then hydrolyzed with 6 N HCl at 110°C for 3 h and lyophilized twice. The hydrolyzate was dissolved in a buffer composed of acetic acid, pyridine, and water in a ratio of 50:5:945 (v/v/v) at pH 3.5 and mixed with standard phosphoserine, phosphothreonine, and phosphotyrosine and spotted on TLE cellulose plate. The phosphoamino acids were separated by electrophoresis at 800 constant voltage for 45 min in the same buffer. The standard phosphoamino acids were then stained with ninhydrin and the plate was autoradiographed.

Construction of mutant P plasmid

Mutant P plasmid was constructed by the revised megaprimer polymerase chain reaction (PCR) method (Barik and Galinski, 1991). pET3a-PC, the plasmid containing the P gene of CHP virus was used as the template in all PCR amplification. The method required three oligonucleotide primers and two rounds of PCR performed on a cloned gene template which will be mutated. Two of these three primers are the terminal primers and the rest represent the internal mutant primer containing the desired base changes. CHPI and CHPII are the two flanking primers containing the terminal sequence. The 27-mer oligonucleotide CHPIII which contained the desired base changes covering a 172- to 198-bp region of the P gene was used as the internal primer having sequence 3′CTCCTTCTTCTACGACTC CTTCTACTA5′. In the first PCR CHPI and CHPIII were used as the primers at an amount of 300 ng in a standard 100-μl reaction mixture containing 100 ng of pET3a-PC as the template and the other components. The PCR was run for 30 cycles with δ the cycle parameter 94°C, 1 min (denaturation); 55°C, 2 min (annealing); 72°C, 1 min (elongation). The reaction mixture was incubated at 95°C for 6 min prior to the PCR for complete denaturation of the template and for 10 min at 72°C after the PCR for complete elongation. A 207-bp-long megaprimer was eluted from agarose gel and the total product was used in the second PCR using CHPII as the another primer and 2 μg of pET3a-PC as the template. The cycle parameter of the second PCR was the same as the first one. The 900-bp full-length product was eluted from the gel and checked by restriction digestion. The PCR product was digested with *Eco*RI and ligated with *Eco*RI-digested pET3a-Pc. The clone was completed by ligating the 1700-bp *Pst*I fragment from pET3a-PC with the *Pst*I digested construct containing the *Eco*RI-digested PCR product. The construct pET-PCM was sequenced to confirm mutation.

Gel filtration analysis of P protein

Bacterially expressed purified P protein at a concentration 0.1 mg/ml was chromatographed through a Sephacryl S-300 gel filtration column equilibrated with transcription reconstitution buffer. An 125-ml bed volume column was first equilibrated with the above mentioned buffer and calibrated with standard proteins of known molecular size, e.g., catalase (242 kDa), yeast alcohol

dehydrogenease (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The protein in transcription reconstitution buffer was fractionated, and the elution profile was monitored by measuring A_{280} of the alternater fractions. P protein was identified by SDS–PAGE, followed by silver staning. In another set, 10 μg of P protein was phosphorylated in 100 μl phosphorylation buffer using [γ^{32}-P]ATP and fractionated similarly in the same column. The elution profile was monitored by measuring the radioactive count of the alternate fractions and P protein was identified by SDS–PAGE followed by autoradiography.

Western blotting

Protein samples analyzed in 10% SDS–PAGE was transferred in PVDF membrane using Bio-Rad semidry transblot apparatus. The membrane was blocked in 5% nonfat dry milk. Hybridization was performed with anti-phosphoserine and anti-phosphothreonine monoclonal antibody from Sigma (Cat. Nos. P 3430 and P3555). The hybridized bands were visualized by HRP-conjugated secondary antibody and diaminobenzidine. In case of hybridization with CHP virus P protein antibody, affinity-purified antibody raised in rabbit against bacterially expressed protein was used.

RESULTS

Role of CKII-mediated phosphorylation in the function of P protein

To investigate the effect of the apparent changes of P protein brought about by CKII-mediated phosphorylation on its function, the bacterially expressed phosphate free and the phosphorylated and gel-eluted P proteins were tested for their activity in *in vitro* transcription mixture reconstituted with L protein and N-RNA template purified from virus. The reaction was carried out according to the procedure mentioned in the methods and spotted on Whatman DE81 paper and washed several times with 0.5 M sodium phosphate buffer. The percentages of radioactive incorporations are presented in the Fig. 1, showing very low counts in the control experiment containing bacterially expressed P0 protein. But a considerable radioactivity was incorporated in the reaction containing the viral as well as the CKII-phosphorylated P protein. To show the direct effect of CKII on the activity of P protein, 0.01 mU of recombinant CKII was used in the *in vitro* transcription reaction along with P0. Figure 1 showed a similar increment of radioactivity as evident in case of P1 with respect to the control containing only CKII. Administration of heparin, a potent CKII inhibitor at a concentration of 2 μg/ml (final concentration) in the reaction mixture inhibited the radioactive incorporation showing the essential role of CKII-mediated phosphorylation in the function of P. Heparin at the concentration used in this experiment did not inhibit the transcription that was shown in the reaction containing viral P protein.

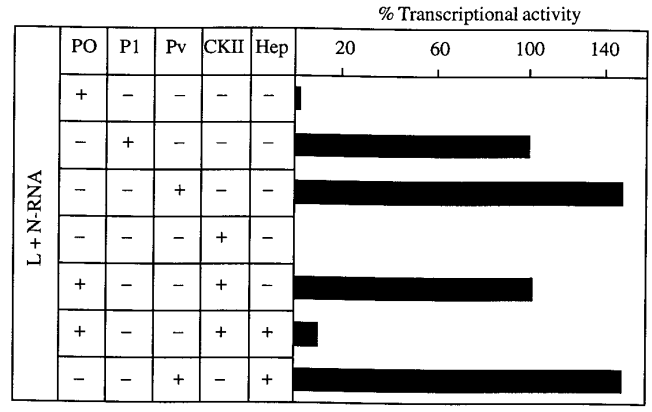

FIG. 1. Transcription reconstitution with the different forms of P proteins. *In vitro* transcriptions were reconstituted using CsCl-banded N-RNA template and cellular kinase free L protein along with unphosphorylated (P0), phosphorylated, gel purified (P1), and viral P protein. CKII and Hep denote recombinant casein kinasell and heparin, respectively. DE81 adsorbable counts were measured and percentages of incorporations were represented taking the count for P1 as 100%.

Stoichiometry of P protein phosphorylation by CKII

The essential role of CKII-mediated phosphorylation in the function of P protein prompted us to detect the number of residues of P protein phosphorylated by CKII. Two different methods had been applied to determine the stoichiometry of phosphorylation. One method was a kinetic experiment of phosphorylation according to Buczylko *et al.* (1991) by taking a known amount of P0 protein (2 μg) and phosphorylating it with an excess amount of (0.02 mU) CKII in presence of [γ-^{32}P]ATP. Five microliters of each aliquot was removed from the reaction mixture at different times up to 1 hr and spotted on Whatman P81 paper and radioactive count was taken after washing those with 5% TCA containing 75 mM phosphoric acid. CKII is an autokinase. So a parallel reaction was set up containing the same amount of CKII without P protein. Aliquots were removed from the reaction mixture at the same time as above and the radioactive count was deducted from the count in the above reaction. Radioactive counts were plotted against time and the stoichiometry was calculated from the saturation value. The stoichiometry calculated was 0.895 molecules of phosphate per molecule of P protein.

In another method, different amounts of P0 protein (0.4, 0.8, 12, 1.8, and 2 μg) were phosphorylated with an excess but same amount (0.02 mU) of CKII for 1 h. Half of the reaction mixture was spotted on Whatman P81 paper, washed, and radioactive count taken. The radioactive count for one concentration of P protein was subtracted from the radioactive count for the next higher

concentration of P protein. The calculation was based on the average subtracted count obtained. The stoichiometry of phosphorylation calculated from this method was 0.875 molecules of phosphate per molecule of P protein. Following both the methods the average stoichiometry of phosphorylation of P protein by CKII was found to be 0.885, i.e., equivalent to 1. The same result was obtained when repeated with uninfected BHK 21 cell extract as the source of kinase. One can not rule out the possibility of misfolding of P protein due to bacterial overexpression which may expose reduced number of CKII on the surface of the protein. But the results of the previous experiments demonstrated that this *in vitro* phosphorylation is sufficient to confer the biological activation of the protein *in vitro.*

CKII phosphorylates P protein is a serine residue

CKII is a Ser/Thr-specific protein kinase. It phosphorylates its substrates to those Ser/Thr which are followed by an acidic amino acid(Glu/Asp) at the +3 position (Pinna, 1990). There are five such residues, three serines, and two threonines in the P protein which can act as potential sites for CKII-mediated phosphorylation (Masters and Banerjee, 1987). Among those five residues only one residue is phosphorylated by CKII as found in the previous experiment. To distinguish whether serine or threonine is the phosphate acceptor residue, the phosphorylated P protein was subjected to Western blot with monoclonal antibodies against phosphoserine and phosphothreonine (Fig. 2a). The result indicated that serine, not threonine, is the phosphoacceptor residue. To confirm the result, phosphoamino acid analysis was done with the *in vitro* phosphorylated protein according to the procedure mentioned under Materials and Methods. The result (Fig. 2b) confirmed that serine is the phosphorylated residue.

FIG. 2. (a) Western blot of phosphorylated P protein with phosphoserine and phosphothreonine antibody. Same concentration of phosphorylated protein samples were analysed in duplicate in SDS–PAGE and subsequently western blotting was performed with monoclonal antibodies against phosphoserine and phosphothreonine seperately. (b) Phosphoaminoacid analysis of the phosphorylated P protein. P protein was phosphorylated by CKII and [γ-^{32}P]ATP, subsequently hydrolyzed by acid and analysed by TLE and autoradiographed. The positions of the standard phosphoamino acids are visualised after staining with ninhydrine and marked. S, T, and Y stand for standard phosphoserine, phosphothreonine, and phosphotyrosine.

Ser^{62} is the putative phosphate accepting site

CKII phosphorylates the VSV(NJ) P protein in ser^{59} and ser^{61} and the VSV(IND) P protein in ser^{60}, thr^{62}, and ser^{64} residues (Takacs *et al.,* 1992; Gao and Lenard, 1995). Comparison of the sequences of CHP virus P protein (Masters and Banerjee, 1987) with its counterparts in the two serotypes of VSV reveals that there is only one residue (ser^{62}) in the corresponding region which can act as a potent CKII phosphorylation site (Fig. 3A). CKII phosphorylates that ser/thr, which is followed by at least an acidic residue at +3 position and presence of acidic residues at the N-terminal side of the site increases the rate of phosphorylation as evidenced by the drop in K_m (Pinna, 1990). Sequence surrounding the ser^{62} of CHP virus P protein shows that it is preceeded by four and followed by five acidic amino acids (Fig. 3A). Consecutive occurence of nine acidic amino acids is unique in the rhabdoviral P protein and no other P protein possess such a suitable CKII phosphorylation site like ser^{62}.

The sequence of the P protein shows 26 digestion sites of trypsin after complete proteolysis which will result in a number of peptide fragments ranging from 6.5 kDa to 220 Da, the second highest being around 2.5 kDa. Five micrograms of P protein was phosphorylated in presence of [γ^{32}-P]ATP and denatured by boiling in the presence of 0.1% SDS. The denatured protein was subjected to proteolysis with 1 μg/ml of trypsin for 16 h and analyzed by SDS–Tricine–polyacrylamide gel followed by autoradiography. The autoradiograph showed a partial digestion pattern after 2 h of digestion which resulted in a single phosphorylated band at around 6 kDa after complete digestion. It appeared from the gel (Fig. 3B) that the peptide lighted up was the longest tryptic-digested polypeptide of calculated molecular mass of 6.5 kDa which covers the region between the 45th and 103rd residues, and this region contains only one CKII phosphorylatable serine which is the ser^{62}. All of these data together prompted us to consider the ser^{62} to be the putative candidate for site-directed mutagenesis.

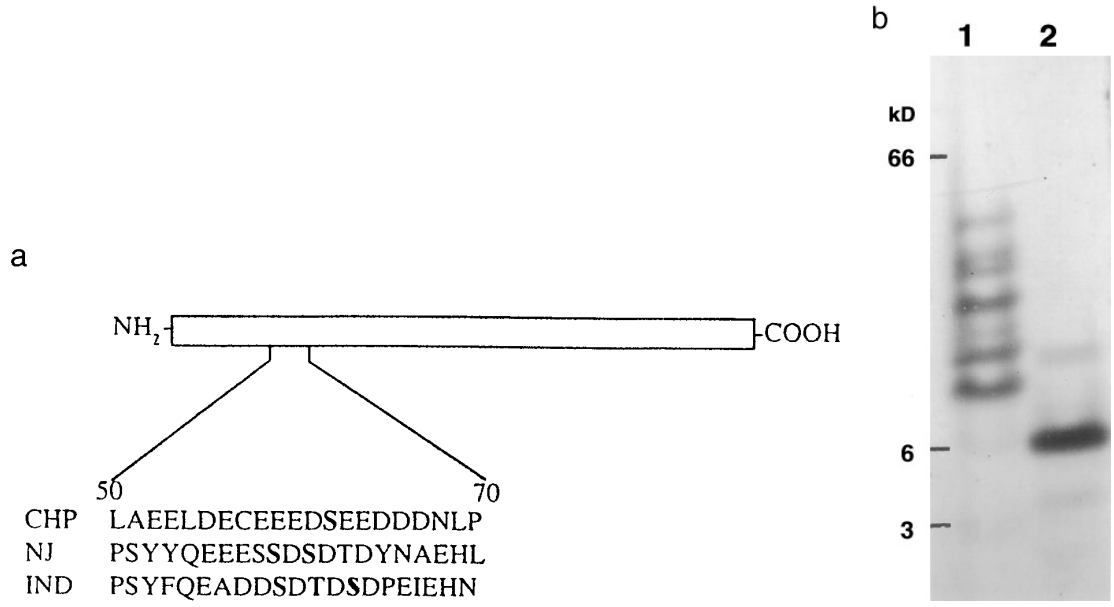

FIG. 3. (a) Sequence comparison of aminoacids (50–70th) of the P proteins of CHP virus and the two serotypes of VSV (NJ and IND). Phosphorylation sites of the VSV (NJ and IND) P proteins and the putative phosphorylation site of CHP virus P protein were indicated by bold letters. (b) Identification of the phosphorylated peptide(s). The ^{32}P-labeled phosphorylated P protein was digested partially (lane 1) and completely (lane 2) with trypsin as mentined in materials and methods.

Identification of phosphorylated residue by alanine substitution

To confirm whether the ser[62] is the target for cellular CKII, a mutant P plasmid was constructed that contained an alanine residue at the 62nd position in place of serine. The mutant plasmid was constructed as described under Materials and Methods. A *Dde*I site (C/TNAG) was created in the internal primer CHPIII using codon bias keeping the amino acid the same to check the mutation primarily. The mutated P gene after the 2nd PCR was cloned in pET3a expression vector as mentioned under Materials and Methods and expressed in *E. coli* and purified as in the case of wild-type P protein. An equal amount of wild-type and mutated P protein was used as the substrate for phosphorylation by CKII in a standard kinase reaction *in vitro.* The result shown in Fig. 4 indicates that the mutant P was phosphorylation negative. Similar results were obtained when uninfected BHK21 cell extract or rabbit reticulocyte lysate was used as a source of host kinase(s). The transactivation ability of the mutant P protein was checked by using it in the *in vitro* transcription reaction along with the bacterially expressed wild-type P protein and P protein isolated from virus as control. Bacterially expressed wild-type P protein was phosphorylated with CKII and purified by gel filtration and used in the reaction. CKII was added in the reaction mixture containing mutated P protein. The autoradiograph in Fig. 5 shows no transcription product in the reactions containing unphosphorylated wild-type P protein, mutated P protein, as well as in the control, demonstrating that serine[62] must be present and phosphorylated for the P protein to be able to transactivate the reconstituted CHP virus transcription system. The bands corresponding to N transcript in lanes 3 and 5 were only about 1 and 3% of the intensity relative to lane 2 and presumably due to the contaminating viral P protein in the L preparation.

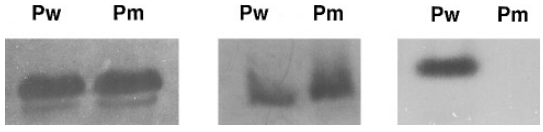

FIG. 4. Phosphorylation analysis of wild type and mutant P protein. Recombinant wild-type and mutated P proteins purified form bacteria were tested as substrate for phosphorylation by recombinant CKII as described in Materials and Methods. Panels 1, 2, and 3 represent the Coommassie staining, Western blot, and autoradiograph of the SDS–polyacrylamide gel containing the wild type (Pw) and mutant (Pm), respectively.

Phosphorylated P protein was eluted at the position of its dimer in the gel filtration

P protein of both the serotypes of VSV have been shown to form homomultimers after CKII-mediated phosphorylation (Das *et al.,* 1995a; Gao and Lenard, 1995). The P protein of VSV (NJ) was eluted at a position of dimer in the gel filtration and the same protein from IND serotype was shown by His-tag dilution method to form transcriptionally active homotrimer. To investigate the situation in the case of P protein of CHP virus, the bacterially expressed protein was subjected to gel filtration before and after phosphorylation. The P protein at a concen-

FIG. 5. Transcription activity of wild-type and mutant P proteins. *In vitro* transcription reaction was reconstituted as described in the legend to Fig. 1, analyzed by 5% polyacrylamide–7 M urea gel, and autoradiographed. The corresponding viral mRNAs were indicated. 0.01 mU of recombinant CKII was added in the reaction containing Pm.

tration of 100 μg/ml in transcription buffer was chromatographed in a Sephacryl S-300 gel filtration column preequilibrated with the same buffer and calibrated with the standard marker protein mentioned under Materials and Methods. Fractions were analyzed by SDS–PAGE and the proteins were identified by its characteristic mobility (M_r ~53,000). When the elution profile was compared with that of the standard proteins the plot showed that the P protein was eluted at a position with a relative molecular mass of 32 $\pm$ 2 kDa, suggesting the monomeric form of the unphosphorylated protein. The P protein at this concentration did not show any natural propensity to form higher migrating nonspecific aggregate. The P0 protein at a concentration of 100 μg/ml was phosphorylated in a 100 μl of standard *in vitro* phosphorylation reaction in presence of [γ-^{32}P]ATP and subjected to gel filtration as above in the same column. Interestingly P1, the phosphorylated form was eluted at the same position as BSA, used as a marker protein, i.e., at a position consistent with its dimeric form (Fig. 6). The fractions were checked by SDS–PAGE followed by autoradiography. We have not checked whether any P molecule, which remained unphosphorylated, was fractionated as monomer or not. We also checked the migration of the mutant after CKII incubation in gel filtration and that behaved like the unphosphorylated form (data not shown). From the above result it appears that P protein of CHP virus may form homodimer after CKII-mediated phosphorylation. However, migration of a protein in gel filtration depends on the Stoke's radius of the molecule. So other sensitive methods of measuring molecular mass can make any confirmed comment.

DISCUSSION

From one and a half decades of extensive investigation the significant observation about the P protein of rhabdovirus that has come into the limelight is that the acidic domain of the protein deserves special attention. Using the bacterially expressed P protein of VSV it has been demonstrated that the acidic N-terminal half is responsible for the two most important characteristics of the protein, acidity and phosphorylation (Banerjee, 1987). This part of the protein could be functionally substituted by the acidic protein tubulin and also after substitution of two basic amino acids by alanine it can be fused with the DNA binding domain of GAL4 to get transcription-activator-like function (Chattopadhyay and Banerjee, 1988; Takacs *et al.,* 1991). It has been demonstrated by us and other groups that acidity of the N-terminal part of P protein is also responsible for its abnormal migration in the SDS–polyacrylamide gels (Banerjee, 1987; Chattopadhyay and Chattopadhyay, 1994). P proteins of several negative-sense RNA viruses have been found to be essentially phosphorylated by host-derived kinases (Barik Banerjee, 1992a; Mazumder *et al.,* 1994; Gao and Lenard, 1995; De *et al.,* 1995; Das *et al.,* 1995b). Recent investigations also suggest that a homomultimerization occurs after host kinase-mediated phosphorylation at its N-terminal half (Das *et al.,* 1995a, Gao *et al.,* 1996) and that is essential for the association of the protein with the transcription complex (Gao and Lenard, 1995). All these results suggest a structural importance of these parts of the protein.

We are interested to study the role of host kinase in the activation of P protein of CHP virus, which is distantly related to VSV. We have used the bacterially expressed P protein as the substrate that we have shown is unphosphorylated, and it is phosphorylated by cellular casein kinase II *in vitro* (Chattopadhyay and Chattopadhyay, 1994). We have determined the biological activity of the phosphorylated and unphosphorylated P protein in the reconstituted transcription reaction. The *in vitro* result shows that the phosphorylated form of the P protein is

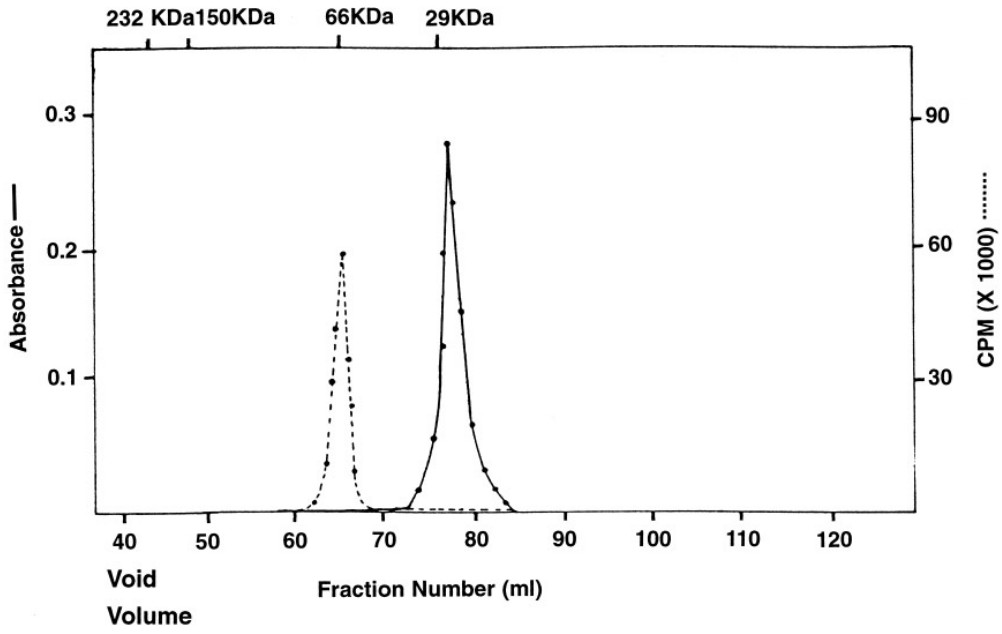

FIG. 6. Gel filtration profile of the P protein. Bacterially expressed unphosphorylated P protein (100 μg/ml) was fractionated through Sephacryl S-300 column as described under Materials and Methods. A_{280nm} of the alternate fractions were plotted (—) against the elution volume. Elution profile (---) of ^{32}P-labeled phosphorylated P protein (100 μg/ml) was fractionated in the same column. The profile was monitored by ^{32}P counting. Positions of the standard markers were mentioned above.

active. Analysis of the sequence shows there are five possible CKII phosphorylation sites on the P protein. In the process to identify the site(s) of phosphorylation we have determined the stoichiometry of CKII-mediated phosphorylation. We have measured it in two different ways and in both the processes the result is the same; i.e., P protein appears to incorporate only one molecule of phosphate when incubated with CKII *in vitro.* Western blot with monoclonal antibody against phosphoserine and phosphothreonine and phosphoamino acid analysis shows the phosphorylated amino acid is serine not threonine. To confine our search we have compared the phosphorylation sites on the P proteins of both the serotypes of VSV with the sequence of CHP virus P protein. The comparison indicated that the ser^{62} was the only putative site for phosphorylation. This idea was further supported by the relative hydropathicity plot, probable secondary structure plot, analysis of the sequence surrounding the ser^{62} (Masters and Banerjee, 1987) and finally by complete digestion with trypsin. Substitution of ser^{62} by alanine prevents the CKII-mediated phosphorylation, and subsequent transcription-supporting activity of P protein demonstrated that CKII-mediated phosphorylation takes place only at ser^{62} and that is necessary and sufficient for the biological activation of P protein *in vitro.* Although P-IND has two sites for phosphorylation, kinetics data suggested that one may suffice for the activity (Gao and Lenard, 1995). It has been demonstrated earlier in the case of VSV(IND)P protein that substitution of one of the phosphorylation sites (ser^{60}/thr^{62}) by aspartic acid imparts a partial transactivation property (Gao and Lenard, 1995) that normally occurs after phosphorylation. This situation naturally occurs in the case of CHP virus P protein where ser^{62} is preceeded by four and followed by five acidic amino acids. However, in contrast we did not observe any transcriptional activation by the unphosphophosphorylated form of P protein. Similar to VSV (NJ) P protein, CHP virus P protein was also eluted at a position of dimer in the gel filtration and changed its alpha-helical content after phosphorylation (data not shown). In the case of P-NJ, substitution of the phosphoacceptor serines with glutamic acid restored the activity of P (De *et al.,* 1997). A particular ionic nature is probably needed for the protein to confer its biological activity and for CHP virus P protein, phosphorylation of ser^{62} appears to be critical to attain that specific ionic nature.

Altogether the results reconcile fairly well with the concept that CKII facilitates the P protein to conform to a precise three-dimensional shape by phosphorylation and thus favors its *in vitro* transcriptional activation property. So phosphorylation by CKII (and also probably by other independent protein kinases) would represent a constitutive posttranslational modification affecting all the newly synthesized P protein molecules after viral infection. Detail studies would certainly shed light to understand the role of P protein phosphorylation and the potential conformational changes coupled with transcription-supporting activity.

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