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N-terminal region of P protein of Chandipura virus is responsible for phosphorylation-mediated homodimerization

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The phosphoprotein P of Chandipura (CHP) virus, an Indian isolate of rhabdovirus, was found to support transcription upon phosphorylation by casein kinase II (CKII). A phosphorylation-induced change in the protein conformation was found to occur at the N-terminal region of the protein. Biochemical studies for further characterization of this phosphorylation-based conformational alteration demonstrated that phosphorylation leads to the transition from an 'open' to 'closed' structure of the protein. The phosphate group introduced by CKII was found to be resistant to phosphatases. This phosphorylation-based structural alteration changes the accessible hydrophobic surface area of the protein and also the available digestion sites of different proteases. The phosphorylated form of P protein was found to be a dimer by His-tag dilution assay. Using the same approach it was found that the N-terminal 46 amino acids are responsible for P–P dimerization, only after phosphorylation.

Keywords: Chandipura virus/dimerization/phosphoprotein/phosphorylation/transcription

Introduction

Chandipura virus (CHP), a negative stranded RNA virus, was first isolated in India in 1965 from serum of an adult human female suffering from atypical dengue (Bhatt and Rodrigues, 1965). CHP virus is a species in the genus *Vesiculovirus* of the family Rhabdoviridae of the order Monomegaloviridae. Classification of this virus as a vesiculovirus is based on its bullet-shaped morphology and comparison of protein and nucleic acid composition with established members of this group, such as vesicular stomatitis virus (VSV). Unlike other rhabdoviruses, CHP enjoys a wide host range including vertebrates and insects. From the available evidence it is fairly clear that mammals may represent dead-end hosts. Mosquitoes, mainly *Ades agepti* and *Ades albipictus*, serve as the vectors between mammals and nature (Ramachandra Rao *et al.*, 1967).

Analysis and characterization of 50 different temperature-sensitive (ts) mutants of CHP virus resulted in the identification of six complementation groups. The intragroup complementation observed among the CHP virus ts mutants suggested that the functional form of at least one of the virion proteins of CHP virus is a multimer (Gadkari and Pringle, 1980a,b).

The 11 kb long genomic RNA, encapsidated by nucleocapsid protein (N-RNA), serves as the template for both replication and transcription (Masters and Banerjee, 1987). Transcription of this genome by viral encoded RNA polymerase produces one leader RNA of 49 bases along with five capped and polyadenylated messenger RNAs which code for five different structural proteins, the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large protein (L) in sequential order and in decreasing amounts. The active transcriptional regime can be carried in a defined system consisting of three viral components: the template (N-RNA), RNA-dependent RNA polymerase (L) and the phosphoprotein (P).

The P protein (32.5 kDa) from both virion and infected cells is always in the phosphorylated form. The phosphoprotein P of CHP virus has been studied in great detail in recent years in our laboratory, mostly regarding its role in transcription. Comparison of the amino acid (aa) sequence of the CHP virus P protein and VSV (New Jersey and Indiana serotypes) revealed only 21% similarities with no consecutive stretches of more than four amino acids being identical between them. The P protein of CHP virus (293 aa) is longer than that of the VSV (NJ) strain (274 aa) and their sites of phosphorylation are also different (Masters and Banerjee, 1987; Chattopadhyay *et al.*, 1997). These apparent dissimilarities in CHP virus P protein and its ability to infect human prompted us to study the protein in greater detail. The simple composition and complex behaviour of P protein make it a very good candidate for structure–function studies.

In different rhabdoviruses the P protein is found to be present in different phosphorylated forms in the matured virion particle (Barik and Banerjee, 1992b). Transcription activation of P has been claimed to be a two-step process. First the unphosphorylated P undergoes phosphorylation by a host kinase to form the protein P1 which then undergoes phosphorylation again by L-associated kinase (LAK) to form P2 (Barik and Banerjee, 1992b); However, according to Gao and Lenard (1995a), this second phosphorylation may also be associated with a kinase of host origin. It has been found that the phosphorylation by the host kinase is sufficient for the transcription *in vitro (*Barik and Banerjee, 1992a).

Previous studies in our laboratory demonstrated that unphosphorylated recombinant P protein expressed in bacteria was transcriptionally inactive. Activation required phosphorylation by a cellular protein kinase which was identified as casein kinase II (CKII) (Chattopadhyay and Chattopadhyay, 1994). Only CKII and no other protein kinases, viz. PKC and PKA tested *in vitro*, phosphorylates the bacterially expressed P protein (data not shown). The stoichiometry of phosphorylation *in vitro* reaches a maximum value of 1 mol of phosphate per mole of P protein. Loss of transcription-supporting ability of P protein on substitution of Ser62 by Ala showed that

phosphorylation of Ser62 is necessary for transcription *in vitro* (Chattopadhyay *et al.*, 1997). The mutant P protein (Ser62–Ala), when cloned in a eukaryotic expression vector under CMV promoter and expressed in VERO cells, was found to be unphosphorylated, whereas under similar condition the wild-type P protein undergoes phosphorylation (data not shown). All these observations conclusively prove that only CKII phosphorylates the P protein and this phosphorylation at Ser62 is essential and sufficient to confer full transcriptional activity.

The role of different cellular kinases is established in the transcription activation property of the phosphoprotein of the related VSV (Chattopadhyay and Banerjee, 1987). However, the mechanism of phosphorylation-dependent transcription activation is still an enigma. Using different biophysical approaches we have proved that there is a change in the protein conformation at the N-terminal half of the P protein of CHP virus after phosphorylation (Raha *et al.*, 1999). Gel filtration studies showed that the *in vitro* phosphoryated protein elutes at the position of dimer whereas the unphosphoryated protein elutes as monomer from the column with respect to its molecular weight (Chattopadhyay *et al*., 1997). But the elution volume cannot be taken as a measure of accurate molecular weight as changes in shape also affect precise elution position.

In this work we explored the nature of the phosphorylation-dependent conformational alteration of the three-dimensional structure of the phosphoprotein P of CHP virus. We approached this question by measuring the change in the hydrophobic character of P protein after phosphorylation. We also monitored the conformational change due to phosphorylation by partial proteolytic digestion followed by SDS–PAGE separation of the peptide fragments to generate a 'fingerprint' that is the characteristic of a particular protein substrate having different tertiary structures. Recently, in the case of VSV (IND), the phosphorylated P protein was shown to be a trimer by His-tag dilution assay (Gao *et al.*, 1996). Using the same approach, we have shown that P protein of CHP virus is a homodimer. Using different deletion mutants of CHP virus P protein, we have also proved the involvement of an N-terminal CKII-modified region in P–P homodimerization. Finally, we conclude that P protein, expressed in a bacterial system, did not dimerize although it contains the N-terminal 1–46 amino acid residues. Hence, upon phosphorylation, the change in the N-terminal end conformation helps the P protein to undergo dimerization.

Materials and methods

Bacterial expression and purification of untagged P protein

Escherichia coli BL21(DE3) was transformed with pET3aPC plasmid containing the P gene. Cells containing the appropriate plasmid were grown in a medium containing 100 μg/ml ampicillin and 0.2% glucose at 25°C. At $O.D._{590}$ = 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Cells were harvested and lysed with lysozyme in 50 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 in the presence of protease inhibitors and then treated with DNase. The lysate was centrifuged to remove the debris and supernatant was used for purification of P protein.

Purification of the untagged P protein from the lysate was carried out using a Q-Sepharose (Pharmacia Biotech) column pre-equilibrated with 50 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 and 1 mM EDTA. The protein was eluted at a 300 mM NaCl salt concentration using a 0–500 mM gradient (Chattopadhyay *et al.*, 1997).

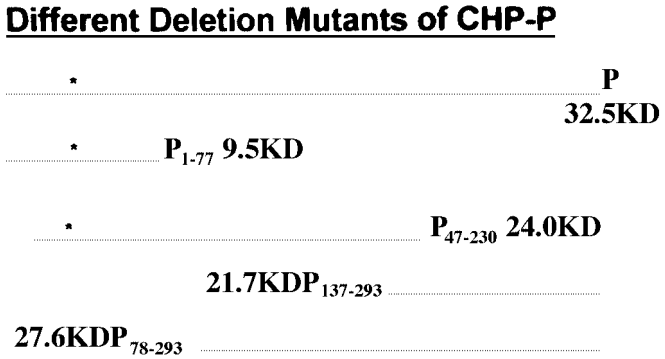

Fig. 1. Schematic diagram of the different deletion mutants of CHP–P protein. The asterisk indicates Ser62, the site of phosphorylation.

Expression and purification of His-tagged P protein from bacteria

The P gene was subcloned in pET15b vector under T7 promoter (Studier *et al.*, 1990). The recombinant protein was expressed as His-tagged at the N-terminal end. The protein was expressed in *E.coli* BL21(DE3) as described earlier and purified through Ni-NTA agarose beads (Novagen). The column was equilibrated with 50 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 and 500 mM NaCl. After charging the protein, the column was washed with the same buffer containing 60 mM imidazole and the protein was eluted with the same buffer containing 1 M imidazole. The eluted protein was dialyzed against 50 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 and 1 mM EDTA to remove imidazole. The tagged protein migrates more slowly than the untagged protein in 10% SDS–PAGE, which is the basis of the His-tag dilution experiment.

Cloning, expression and purification of the different deletion mutants of P protein having N-terminal His-tag

The P protein of CHP virus is 293 amino acids long. From the pET3a-PC clone the P gene was released and different restriction enzyme based deletion mutants of the CHP–P gene were constructed in bacterial expression vectors pRSET A, B, C (Stratagene) keeping the reading frame unchanged. All the mutant P proteins have the N-terminal His-tag. The expressed proteins were simultaneously purified using Ni-NTA agarose beads as mentioned earlier and checked by SDS–PAGE followed by Western blotting with antibody raised against P protein (data not shown). A schematic diagram of the different truncated P proteins is shown in Figure 1. The deletion mutant $P_{1–77}$ contains the N-terminal 77 amino acid residues, which includes Ser62, i.e. the CKII target site. The deletion mutant $P_{47–230}$ contains amino acid residues 47–230 and this mutant also contains Ser62. The deletion mutants $P_{137–293}$ and $P_{78–293}$ contain the amino acid residues 137–293 and 78–293, respectively. Owing to the presence of highly acidic amino acid residues, the $P_{1–77}$ mutant showed abnormal migration in SDS–PAGE.

In vitro kinasing of the recombinant P protein

The purified unphosphorylated P protein (both untagged and tagged and the truncated P proteins) was phosphorylated *in vitro* by human recombinant CKII (Boehringer-Mannheim) in a transcription buffer containing 50 μM ATP for 1 h at 30°C. To check the phosphorylation status of the different

deletion mutants of P protein of CHP virus, the purified deletion mutants were subjected to *in vitro* phosphorylation by CKII under the same conditions.

To check the phosphorylation by LAK, the bacterially expressed P protein was phosphorylated *in vitro* by CKII using cold ATP. The phosphorylation reaction was stopped using heparin. The phosphorylated P protein was then incubated with lysed virus in presence of heparin and [γ^{32}P]ATP.

Sensitivity of the incorporated phosphate group to alkaline phosphatases

A 1 μg amount of recombinant P protein was phosphorylated *in vitro* by CKII using [γ^{32}P]ATP. The reaction was stopped by adding 5 μg/ml (final concentration) of heparin. The reaction mixture was dialyzed against 50 mM Tris–HCl (pH 8.0) for 3 h to remove excess ATP. One part of the reaction mixture was treated with 1 U of calf intestinal alkaline phosphatases (CIAP, GIBCO-BRL) and another part was incubated with 1 U of bacterial alkaline phosphatase (BAP, GIBCO-BRL), both at 37°C for 30 min. The reaction mixtures along with the untreated samples were analyzed by 10% SDS–PAGE followed by autoradiography. To check the activity of the phosphatases, the P protein was phosphorylated with CKII in presence of cold ATP and dialyzed and was subjected to phosphorylation with the viral LAK in the presence of heparin (5 μg/ml final concentration). The purified viral particle was lysed in the lysis buffer containing 10 mM Tris–HCl (pH 8.0), 5% (v/v) glycerol, 0.4 M NaCl, 1.85% Triton X-100, 0.6 mM DTT. After centrifugation the supernatant (5 μl) was used as a source of LAK to phosphorylate the CKII phosphorylated P protein in presence of [γ^{32}P]ATP. After 30 min of phosphorylation, the reaction mixture was divided into four fractions: one part of the sample was mixed with SDS sample buffer to stop the reaction, the second part was incubated at 37°C without any phosphatase and the other two parts were incubated with CIAP and BAP, respectively, at 37°C for 30 min. The samples were analyzed by 10% SDS–PAGE followed by autoradiography.

Binding of P protein to Phenyl Sepharose column

Binding of proteins to a Phenyl Sepharose column matrix gives us an idea of the exposed hydrophobic surface area of the protein. The binding of both the unphosphorylated and phosphorylated P protein to a Phenyl Sepharose gel matrix was determined according to Yamamato (1991). A 25 μg amount of purified P protein (both unphosphorylated and *in vitro* phosphorylated) in 60 μl of binding buffer containing 50 mM Tris–HCl (pH 8.0), 0.1 M NaCl and various concentrations of ethylene glycol were added to 30 μl of a suspension of Phenyl Sepharose equilibrated with the same buffer. Samples were incubated at 4°C for 30 min. The suspensions were centrifuged at 6000 *g* for 1 min and the supernatants were analyzed by 10% SDS–PAGE. The amount of proteins was quantitated by densitometric scanning of Coomassie Brilliant Blue-stained gel in a Pharmacia LKB laser scanner using a calibration curve constructed with known amounts of P protein.

Protein fingerprinting by limited protease digestion

Amounts of 20 μg each of purified phosphorylated and unphosphorylated P protein were incubated at 37°C for 10 min with different concentrations of TPCK-trypsin and chymotrypsin and the reactions were stopped with 50 μg/ml TLCK and TPCK, respectively. The samples were analyzed by 16% SDS–PAGE and stained with Coomassie Brilliant Blue.

His-tag dilution assay

Unphosphorylated P protein and N-terminal His-tagged unphosphorylated P protein were obtained from overexpression of pET3aPC and pET15PC clones, respectively. Untagged protein moves faster than the tagged protein in 10% SDS–PAGE. To a constant amount of tagged protein (1 μg), increasing amount of untagged proteins were added and the mixtures were subjected to *in vitro* phosphorylation by CKII. Multimers possessing at least one tag subunit were recovered through Ni-binding resin. The different sets were analyzed by 10% SDS–PAGE. The proteins in the gel were quantitated by laser densitometric scanning after Coomassie Brilliant Blue staining. Band intensities were related to standard concentrations of tagged and untagged P protein separately. The molar ratios of untagged to tagged protein in the purified complex were plotted against the weight ratios of the untagged to tagged protein in the original mixture.

To identify the region involved in P–P oligomerization, the same experiment was performed in which the His-tagged full-length P protein was replaced with different deletion mutants of P protein having His-tag at the N-terminal end. The phosphorylation status of different truncated P proteins was checked by *in vitro* phosphorylation with CKII. A 1 μg amount of the purified untagged P protein was mixed with the different purified deletion mutants having the same concentration (1 μg). The different mixtures were phosphorylated *in vitro* by CKII and then passed through a column containing Ni-NTA agarose in order to purify the dimer containing at least one tagged subunit as well as the free tagged protein. The samples were then analyzed by 10% SDS–PAGE.

Results

Inaccessibility of the CKII-incorporated phosphate group to phosphatases

To identify whether the CKII-incorporated phosphate group is accessible to alkaline phosphatases, the bacterially expressed P protein was subjected to *in vitro* kinasing by CKII using [γ^{32}P]ATP. The reactions were stopped by heparin followed by incubation with CIAP and BAP. An autoradiogram of the CIAP- and BAP-treated phosphorylated P protein shows the same intensities as the untreated one (Figure 2A, lanes 1–4), which indicates that CKII irreversibily incorporated phosphate group into protein in such a way that it became inaccessible to the phosphatases used in the experiment. As a control, we phosphorylated the P protein by LAK in presence of heparin (to nullify the possibility of phosphorylation by CKII) as mentioned in the Methods section. When we compared the intensities of the radioactive signals in the experimental lane (i.e. CIAP- and BAP-treated samples) with those in the control lane, we found that the radioactive signals decreased (Figure 2A, lanes 5–8). The Coomassie Brilliant Blue-stained gel (Figure 2B) indicates the constant amount of P protein used in the experiment. This indicates that the phosphatases used in this experiment are active and the phosphate group(s) incorporated by LAK can be removed by phosphatases. This result suggests that there might be a conformational alteration of the P protein due to CKII-mediated phosphorylation which in turn hides the CKII incorporated phosphate group so that it is no longer accessible to phosphatases used in this experiment. The recombinant P protein could not be phosphorylated by LAK in the presence of heparin whereas under the same conditions the CKII-phosphorylated P protein could be

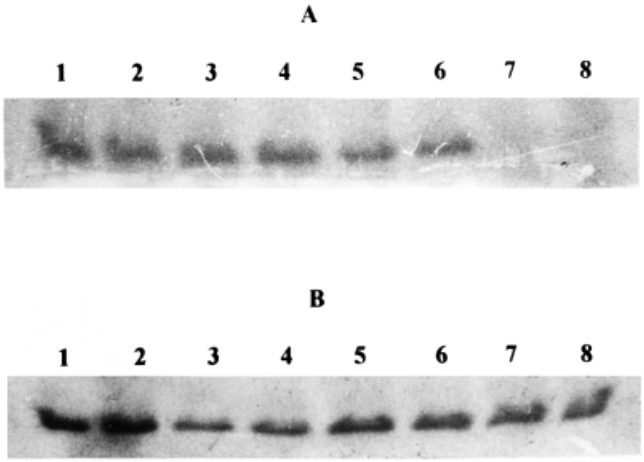

Fig. 2. Effect of phosphatase on the phosphorylated P protein. (**A**) Lanes 1–4: autoradiograph showing the effect of alkaline phosphatases on the *in vitro* phosphorylation of P protein by CKII. The recombinant P protein was subjected to *in vitro* phosphorylation by CKII using [γ^{32}P]ATP followed by phosphatase treatment. Lane 1, control lane (incubated without phosphatase); lane 2, CIAP-treated phosphorylated protein; lane 3, BAP-treated phosphorylated P protein. Lanes 5–8: autoradiograph showing the effect of alkaline phosphatases on the *in vitro* phosphorylation of P protein by L associated kinase. The bacterially expressed purified P protein was subjected to *in vitro* phosphorylation by CKII using cold ATP. The reaction was stopped by adding heparin, a CKII inhibitor, and the phosphorylated protein was further incubated with viral lysate (source of LAK) with [γ^{32}P]ATP. Lane 5, P protein phosphorylated by LAK; lane 6, phosphorylated P protein incubated at 37°C without any phosphatase; lane 7, CIAP-treated phosphorylated protein; lane 8, BAP-treated phosphorylated P protein. (**B**) Coomasie Brilliant Blue-stained 10% SDS–PAGE showing the amount of P proteins used in the above experiments. Lanes 1–8 correspond to lanes 1–8 in (A).

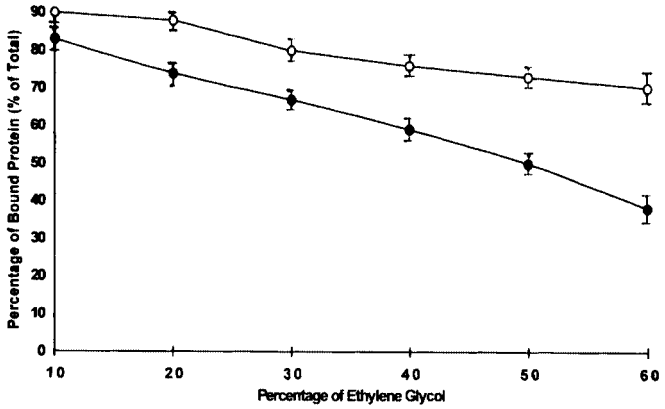

Fig. 3. Binding of unphosphorylated (●) and phosphorylated (○) P protein to the Phenyl Sepharose column. A 25 µg amount of purified P protein (both unphosphorylated and *in vitro* phosphorylated) in 60 µl of binding buffer containing 50 mM Tris–HCl, pH 8.0, with 0.1 M NaCl and various concentrations of ethylene glycol was added to 30 µl of a suspension of Phenyl Sepharose equilibrated with the same buffer. Samples were incubated at 4°C for 30 min. The suspensions were centrifuged at 6000 *g* for 1 min and the supernatants were analyzed by 10% SDS–PAGE. The band intensities were quantified by laser densitometric scanning after Coomasie Brilliant Blue staining.

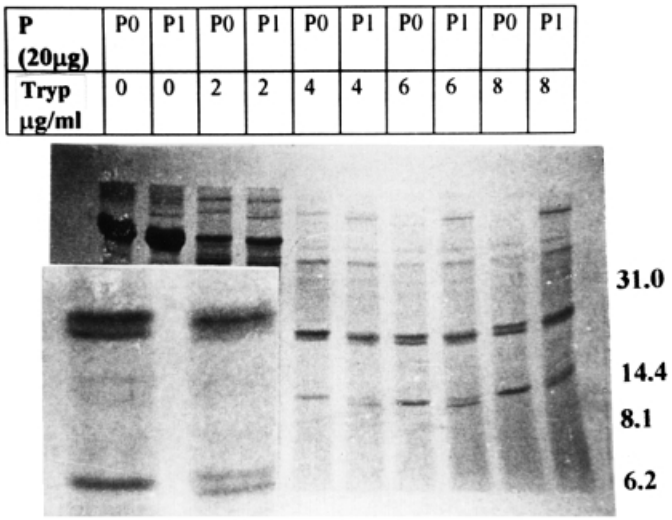

Fig. 4. Coomasie Brilliant Blue-stained 16% SDS–PAGE showing the partial protease digestion pattern of the unphosphorylated (P0) and the phosphorylated (P1) P protein by trypsin. The positions of the standard molecular weight markers are shown. The inset represents a magnified view of the faster migrating bands of the unphosphorylated and phosphorylated P proteins digested with 6 µg/ml trypsin.

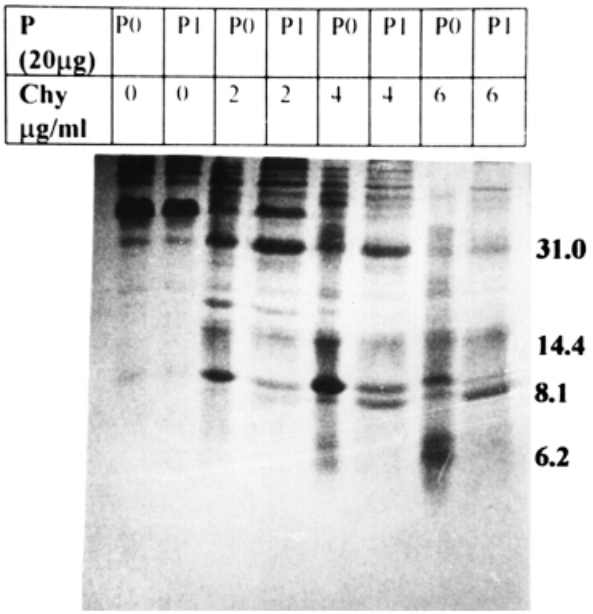

Fig. 5. Coomasie Brilliant Blue-stained 16% SDS–PAGE showing the partial protease digestion pattern of the unphosphorylated (P0) and the phosphorylated (P1) P protein by chymotrypsin. The positions of the standard molecular weight markers are shown.

phosphorylated by LAK. This demonstrates the sequential phosphorylation of P protein by CKII and LAK.

Phosphorylation changes the exposed hydrophobic surface area of the P protein

Proteins bind to the Phenyl Sepharose column matrix as a function of exposed hydrophobic surface area (Yamamato, 1991). Phosphorylation induces a small but significant decrease in the binding of P protein to the Phenyl Sepharose column matrix. Most of the phosphorylated P protein can be eluted at a lower concentration of ethylene glycol (Figure 3). This clearly indicates the decrease in the exposed hydrophobic surface area of P protein after phosphorylation. Hence phosphorylation-induced alteration in the conformation changes the accessible hydrophobic surface area.

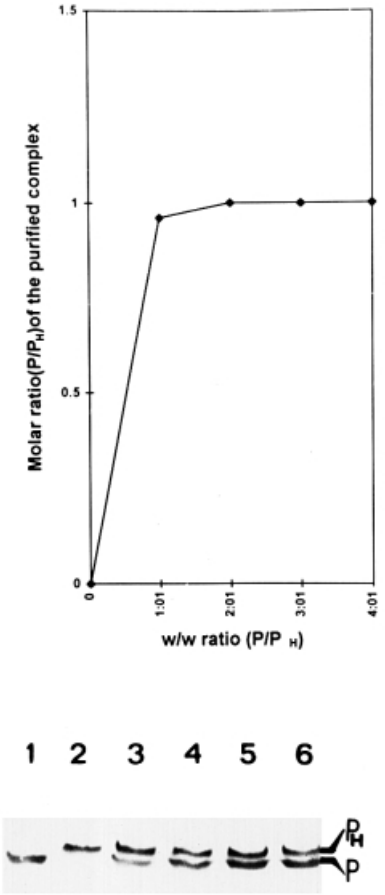

Fig. 6. His-tag dilution assay demonstrating the homodimer formation of the P protein after phosphorylation *in vitro*. The upper panel shows the molar ratio of the tagged to untagged P protein whereas the lower panel shows the Coomasie Brilliant Blue-stained SDS–PAGE of the different samples which were further quantitated by laser densitometric scanning to obtain the molar ratio by comparison with the standard proteins.

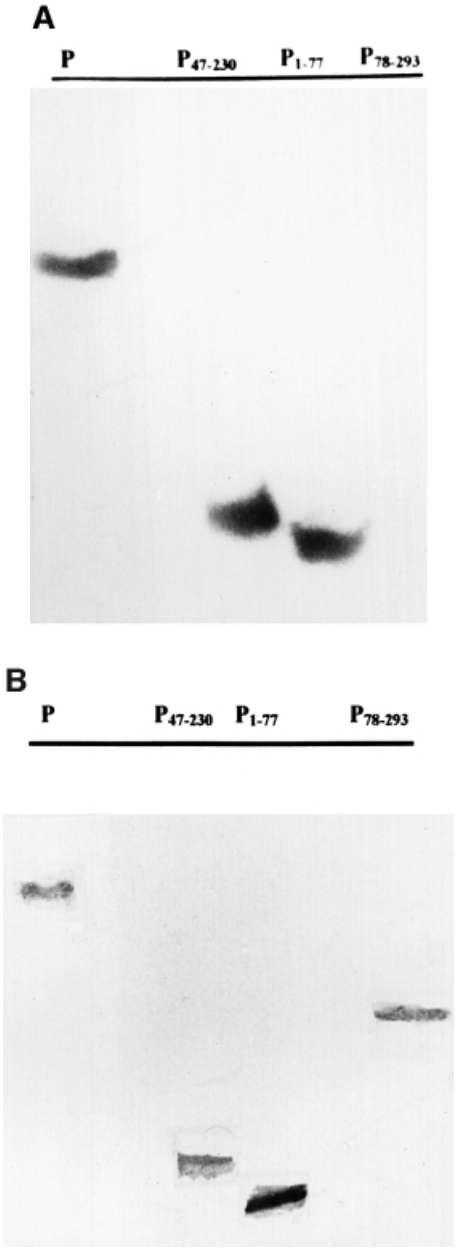

Fig. 7. Phosphorylation status of different deletion mutants of P protein. (**A**) Autoradiograph showing the phosphorylation status of the different deletion mutants of the P protein. The proteins were phosphorylated *in vitro* by CKII and analyzed by 10% SDS–PAGE followed by autoradiography. (**B**) Western blot of the same SDS–PAGE with P_{CHP} antibody to confirm the expression of the different deletion mutants used in this experiment.

Phosphorylation-induced conformational alteration changes the availability of digestion sites of the proteases

Protein fingerprinting can often be used as a very good tool in identifying protein conformational alterations (Calvert and Gratzer, 1978). If the altered three-dimensional structure changes the accessible protease digestion sites, the digestion pattern changes when the proteins are subjected to limited protease digestion. We attempted a similar approach to study the conformational alteration of CHP–P protein by limited protease digestion with trypsin and chymotrypsin. Both unphosphorylated and *in vitro* phosphorylated P proteins were subjected to partial protease digestion with the two proteases.

Partial digestion with trypsin showed changes at two positions as shown in the inset in Figure 4. One peptide migrating around 25 kDa in 16% SDS–PAGE appeared in the unphosphorylated samples when digested partially with 2 μg/ml trypsin. This band was absent in the phosphorylated sample at the corresponding protease concentration but appeared at relatively higher concentration of protease. Another peptide migrating just below the previous one appeared when the unphosphorylated sample was digested with 4 μg/ml trypsin, but no new band appeared at this position when the phosphorylated sample was digested with the same amount of protease. The difference in the pattern at these positions remained unaltered when digested with 6 and 8 μg/ml of trypsin. The situation was different in another region. Only one peptide had appeared in the region around 14 kDa in the unphosphorylated sample, whereas there were two peptides migrating closely in the same region in the phosphorylated sample when digested with a larger amount of proteases (6–8 μg/ml).

Partial protease digestion of unphosphorylated and phosphorylated samples with chymotrypsin showed (Figure 5) two faster migrating peptides, around 10 kDa when digested with 4 and 6 μg/ml of chymotrysin. The relative ratios of concentrations of the two peptides were different in the two samples. In the unphosphorylated sample the upper band was

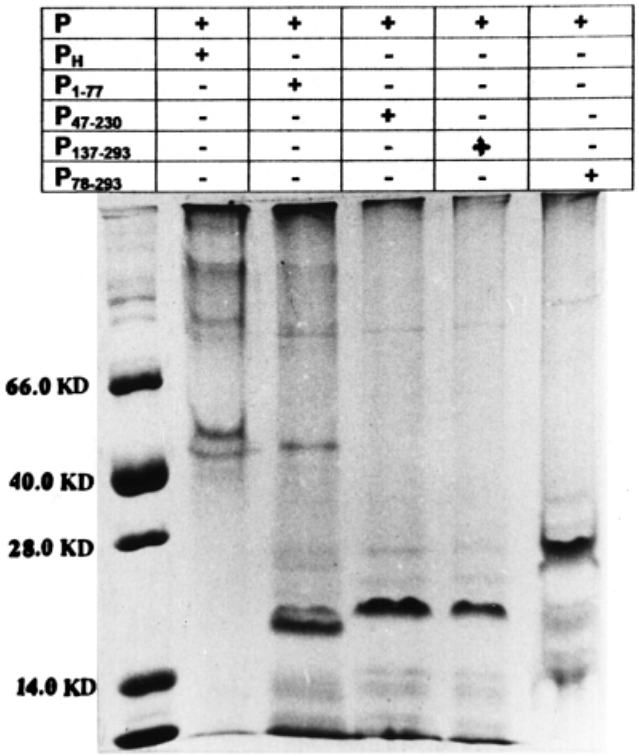

Fig. 8. Modified His-tag dilution assay showing the involvement of the different domains of P protein in homodimer formation. The different deletion mutants used in different sets are given in the upper panel.

more intense than the lower one and the concentration of the upper band increased with increasing concentration of chymotrypsin whereas in the phosphorylated sample initially the intensity of the lower band was low. However, gradually the intensity of the upper band decreased and that of the lower band increased with increase in the chymotrypsin concentration. This result is highly reproducible.

CKII is an autophosphorylating enzyme, but the concentration of CKII used was so low that phosphorylation of CKII did not make any difference to the digestion pattern (not shown).

Phosphorylation induces the dimerization of P protein

In order to determine the stoichiometry of multimerization of P protein, the His-tag dilution method (Gao *et al.*, 1996) was applied. It was found that N-terminal His-tagged P protein could support transcription after phosphorylation with CKII (data not shown). It was also found that the elution profile of the phosphorylated His-tagged protein from the gel filtration column (Sephacryl S-300) corresponds to the oligomeric molecule and was different from the unphosphorylated one. Based on these observations, we used the His-tag dilution assay method to determine the stoichiometry of multimerization.

To a constant amount of the tagged protein, increasing amounts of untagged protein were added and phosphorylated *in vitro*. After recovering the multimer containing the tagged subunit on Ni beads, we analyzed the sample by 10% SDS–PAGE. As untagged protein moves faster than the tagged protein, after Coomassie Brilliant Blue staining we could quantify two types of proteins by laser densitometric scanning. Plotting molar ratio against weight ratio, we obtained the saturating molar ratio value of the untagged:tagged protein as 1:1 (Figure 6), indicating the involvement of one tagged and one untagged subunit in the homomultimer, which supports the formation of a P–P dimer upon phosphorylation *in vitro*. The His-tag dilution assay was also performed by mixing His-tag P and the P protein without phosphorylation and it was found that no untagged P protein could be eluted through the Ni-NTA agarose column, demonstrating that unphosphorylated P could not dimerize (data not shown).

CKII can phosphorylate the deletion mutants containing Ser62 only

Previously the CKII target site was mapped and it was found that Ser62 is the only site for CKII phosphorylation. To check the phosphorylation status of the truncated P protein, the deletion mutants of CHP–P protein were subjected to *in vitro* phosphorylation by CKII along with the P protein as the control. It was found that the $P_{1–77}$ and $P_{47–233}$ proteins were phosphorylated by CKII like the wild-type P protein (Figure 7A and B), whereas neither the $P_{137–293}$ nor the $P_{78–293}$ proteins were phosphorylated under identical conditions. This indicates that CKII is phosphorylating the deletion mutants, which contain Ser62.

The N-Terminal 46 amino acids are involved in P–P dimerization

To determine the role of different regions of the P protein in P–P homodimerization, we performed a modified His-tag dilution assay. In this case, different truncated P proteins having His-tag at the N-terminal end replaced the His-tagged P protein. After *in vitro* kinasing, the complexes containing at least one tagged subunit or the unreacted tagged protein were purified using Ni-NTA agarose and analyzed by 10% SDS–PAGE. From the results (Figure 8), it is clear that only the deletion mutant $P_{1–77}$ protein can form a dimer with the wild-type full-length P protein when phosphorylated *in vitro*. However, the deletion mutants $P_{47–233}$, $P_{137–293}$ and $P_{78–293}$ failed to form a dimer with the wild-type P protein even after incubation with CKII. This indicates the possibility of involvement of the N-terminal 77 amino acid-containing region in P–P dimerization. The *in vitro* phosphorylated deletion mutant $P_{47–230}$ protein fails to dimerize with the wild-type protein, which rules out the possibility of the involvement of the 47–77 amino acid residues in dimer formation. Hence, from the His-tag dilution assay, it is clear that there is no direct involvement of the C-terminal amino acids in P–P dimerization and the N-terminal 46 amino acid residues are important for the formation of the dimer.

Discussion

The role of phosphorylation in viral transcription is fairly well established in different RNA viruses (Banerjee, 1987; Chang *et al.*, 1974, Chattopadhyay *et al.*, 1997). Phosphorylation-induced conformational change has already been reported in the related VSV (NJ) (Das *et al.*, 1995), whereas in case of VSV (IND) no conformational alteration was observed in circular dichroic spectroscopic studies (Gao *et al.*, 1996). In CHP virus our group has already reported the phosphorylation-induced activation of transcription (Chattopadhyay *et al.*, 1997). We also reported the phosphorylation-induced 'unstructured' to 'structured' transition at the N-terminal half of P protein of CHP virus by different biophysical techniques (Raha *et al.*, 1999). Phosphorylation-induced homomultimerization is a common phenomenon in the case of almost all negative stranded RNA

viruses. In the case of VSV (NJ), phosphorylation-induced dimerization was observed (Das *et al.*, 1995), whereas in VSV (IND), the phosphorylated form is a trimer (Gao *et al.*, 1996). In the case of CHP virus, we have previously reported the possibility of the formation of a dimer as evidenced from gel filtration analysis (Chattopadhyay *et al.*, 1997).

The experiments reported in this paper provide further characterization of the phosphorylation-induced conformational alteration of the P protein of CHP virus by some biochemical techniques. We have also conclusively proved the formation of a homodimer of P protein of CHP virus due to phosphorylation. In a previous paper we demonstrated that phosphorylation of P protein by CKII is essential for transcriptional activity (Chattopadhyay *et al*., 1997).

The stability of the phosphate groups of P protein deserves special attention. The first biochemical approach to monitoring the change in the tertiary structure of P protein was the inaccessibility of the CKII-incorporated phosphate group to phosphatases whereas the phosphate group(s) incorporated by LAK can be dephosphorylated by the same phosphatases. We have seen that no phosphorylation of recombinant P protein occurs by LAK in the presence of heparin, a CKII inhibitor (data not shown). Hence phosphorylation by LAK is possible only if P protein is phosphorylated first with CKII. This proves that after phosphorylation by CKII the protein undergoes a change in conformation which then becomes the substrate for another step of phosphorylation by LAK. Although the precise role of second phosphorylation in CHP virus is still unknown, it is clear that the change in the three-dimensional structure of P protein after phosphorylation by CKII alters the structure of P protein in such a way that the phosphate group becomes buried within the core of the protein moiety and thus becomes resistant to phosphatases, whereas in the case of second phosphorylation by LAK the incorporated phospate groups become phosphatase sensitive. The decrease in the binding affinity of the phosphorylated protein to Phenyl Sepharose gives an idea of the decreased hydrophobic surface area upon phosphorylation. Ethylene glycol affects the binding of P protein to Phenyl Sepharose much less in the unphosphorylated form than in the phosphorylated form. All these results together may indicate that phosphorylation induces a change from an 'open' to 'closed' conformation of the protein.

The partial protease digestion patterns demonstrate clearly that there is a change in the tertiary structure and this was reflected in the availability of digestion sites of the proteases. However, it is also noticeable that the overall digestion pattern of the P protein changes only at two or three places although the changes are distinct. Although it is not certain that the peptides migrating at the same position in the case of both the unphosphorylated and phosphorylated proteins are the same peptide, it is conceivable that the changes occurred at the region most probably surrounding the sites of phosphorylation as indicated by phosphatase resistance. However, it does not exclude the possibility that the introduced phosphate group is buried in the monomer–monomer interface in the P–P homodimer and in turn is not accessible to phosphatases and to proteases. The partial protease digestion pattern of unphosphorylated and phosphorylated CHP–P protein also indicates a domain structure of CHP–P protein.

Gel filtration through a Sephacryl S-300 column indicates the possibility of dimerization of unphosphorylated monomeric P protein (Chattopadhyay *et al.*, 1997). Since this type of chromatography does not measure the molecular weight, molecules are separated from each other on these columns based on their shape and size. We can only have an idea of the Stokes radius of the molecule. For instance, if the molecule were a highly 'coiled blob' before phosphorylation and then became an 'extended rod' after phosphorylation, the elution profile may follow the profile obtained in the size exclusion chromatographic results. However, by the His-tag dilution assay it was conclusively proved that phosphorylation induces dimerization which is most probably needed for the transcription activation property of the P protein.

In the case of the P protein of VSV (IND), it was found that the N-terminal 1–30 amino acids are involved in oligomerization (Gao and Lenard, 1995a,b). The computer prediction of the five rhabdovirus P proteins available, VSV-IND, VSV-NJ, rabies, CHP and Piry, indicates that except for Piry virus, all P proteins contain a putative helix of ~30 residues near the N-termini with an elevated coiled-coil potential. In the case of paramyxovirus, a 'coiled-coil' region at the C-terminal end was found to be responsible for oligomerization (Curran *et al.*, 1995). In CHP virus it was reported that phosphorylation induces an 'unstructured' to 'structured' transition in the N-terminal region (residues 49–69) of P protein surrounding the CKII target site (Raha *et al.*, 1999). As evidenced by the His-tag dilution assay, the direct involvement of the N-terminal 46 amino acid residues in P–P dimerization also indicates the role of the domain near the CKII target region, which was previously shown to be responsible for transcription activation (Chattopadhyay *et al.*, 1997). The CKII target region is predicted to be disordered, and upon phosphorylation undergoes a definite change in the tertiary structure that may help the N-terminal coiled-coil region of the P protein to form the dimer. The results reconcile fairly well with the idea that CKII may act as an 'architectural protein' to sculpt the P protein into the precise three-dimensional shape which helps in activation of transcription by the virion RNA polymerase. However, only detailed NMR and crystallographic studies can pinpoint the CKII-mediated dynamic changes at the N-terminal region of the P protein upon phosphoryation to confer the role of phosphoprotein in the viral life cycle.

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