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Novel Binding of GTP to the Phosphoprotein (P) of Vesicular Stomatitis Virus

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The phosphoprotein (P) of vesicular stomatitis virus (VSV) is a subunit of the RNA polymerase (L) that transcribes the negative strand genome RNA into mRNAs both in vitro and in vivo. We have previously shown that the P protein of VSV, expressed in *E. coli*, is biologically inactive unless phosphorylated at specific serine residues by cellular casein kinase II (CKII). In the present study we present evidence that the P protein, in addition to being phosphorylated, binds covalently to GTP only when it is phosphorylated. Competition experiments show that ATP, ADP, GTP, and GDP can compete for the binding site(s) of GTP but not AMP, GMP, CTP, or UTP. Interestingly, once GTP is bound to P protein it cannot be displaced by unlabeled GTP. The GTP binding site has been mapped within the domain where the phosphorylation of P protein by CKII occurs. Finally, we show that phosphorylation negative P mutants P3A (P60A, P62A, P64A), P3E (P60E, P62E, P64E), and P3R (P60R, P62R, P64R) failed to bind to GTP, indicating that phosphorylation of P is indeed essential for binding to GTP. Although the precise role of binding of GTP to P is unclear, it appears that phosphorylation of P may initiate a structural change within the P protein allowing GTP to bind, thus manifesting biological function to the transcription factor.

Vesicular stomatitis virus P protein Phosphorylation GTP binding

THE 29-kDa phosphoprotein (P) of vesicular stomatitis virus (VSV), a negative strand RNA virus, is an essential regulatory component of virion associated RNA polymerase complex (3,4). The phosphoprotein (P) and the 241-kDa L protein constitute the RNA polymerase; they together are needed to transcribe the linear, single-stranded viral RNA genome, which is tightly wrapped with the nucleocapsid (N) protein (N–RNA template) (3,4). Genetic and biochemical studies have suggested that the L protein encodes all the basic transcriptional and mRNA modification activities, whereas the phosphorylated P protein serves as an auxiliary component in both transcription and replication of the genome RNA (3,4,15,32). The N–RNA complex, the P protein, and the L protein, which form the transcribing ribonucleoprotein (RNP) core, can each be separately purified from the virions. Synthesis of mRNAs, which are capped at the 5′ ends and polyadenylated at the 3′ ends, occurs when these components are mixed in vitro (1,2,16,17). In a recent series of experiments we have shown that the L protein expressed by recombinant baculovirus in insect cells is tightly associated with the cellular translation elongation factors EF-1$\alpha\beta\gamma$ (12,27) as well as cellular guanyltransferase (18) as part of the multiprotein subunit complex of the L protein. Thus, the RNA polymerase holoenzyme appears to be comprised of L and phosphorylated P protein and specific host proteins.

Initial studies with the P protein of Indiana serotype isolated from the virions or infected cell lysates indicated that it exists in a variety of phosphorylated states (19,20,22,25,26). Availability of *E. coli*-expressed VSV P in an unphosphorylated form has greatly facilitated the study of the role of phosphorylation of P protein mediated by cellular casein kinase II (CKII) in transcription in vitro (5,6). The CKII-mediated phosphorylation sites in P_{IND} are mapped at Ser 60, Thr 62, and Ser 64 within the acidic domain

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I using recombinant CKII and bacterially expressed P (10). In contrast, using BHK cell extract as the source of CKII or P protein expressed in COS cells, the phosphorylation sites are mapped at Ser 60 and Ser 64, suggesting that T62 phosphorylation in vivo may be sensitive to phosphatase action (10). We further confirmed that P1 (CKII-mediated phosphorylation) is identical to the previously reported NS1 species and P2 (LAK-mediated phosphorylation) is identical to NS2 species; the latter phosphorylation is mapped at Ser 226 and Ser 227 within domain II (10). We carried out a detailed study of the role of phosphorylation in P protein function both in vitro and in vivo. Using an in vivo minigenome transcription/replication system, we demonstrated that phosphorylation in acidic domain I is required for transcription but not for replication of the minigenome (28). On the other hand, the phosphorylation in domain II is shown to be required for replication but not in transcription (21). Thus, the phosphorylated state of P protein seems to be an important determinant in regulating transcription and replication mediated by the L protein.

During our continued effort to study the capping activity of the L and P proteins we searched for the formation of a possible enzyme–guanosine nucleotide complex, which is an obligatory intermediate for the capping reaction (30). We routinely observed binding of GTP with the P protein as part of the RNA polymerase holoenzyme, whereas no such complex was detected with the L protein (13). The exact nature of GTP/P binding was not apparent at that time. We have now studied in detail the GTP binding property of the P protein and in this communication we present evidence that the P protein, in addition to being phosphorylated, covalently binds to GTP only when it is in the phosphorylated state, suggesting a possible role of GTP-bound P in the transcriptive or replicative property of the RNA polymerase holoenzyme.

MATERIALS AND METHODS

Cell Cultures

The recombinant baculovirus BacPAK6-L containing the L gene of VSV was propagated in *Spodoptera frugiperda* cells (IPLB-Sf-21) (27). Sf 21 cells were grown in a monolayer culture at 27°C in TNM-FH medium supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin (27).

Purification of N–RNA Template

The N–RNA template was isolated and purified as described previously (14). Purified VSV (10 mg) was disrupted in a buffer containing 0.4 M NaCl, 10 mM Tris-HCl, pH 8.0, 5% glycerol, 2% Triton X-100, and 1 mM DTT by incubation on ice for 90 min with occasional stirring. The viral RNP 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 $MgCl_2$, and 1 mM DTT for 2 h at 200,000 × *g* in SW60 rotor at 4°C. RNP was collected from the top of the glycerol cushion and was treated with the same buffer containing 1 M NaCl and 0.5% Triton X-100 to dissociate L and P proteins from the N–RNA template. N–RNA was purified by centrifugation through 30% glycerol onto a 100% glycerol cushion in the same manner as described above. N–RNA template was further purified by an additional high salt wash, centrifuged through 15% renographin onto a 76% renographin cushion followed by three serial banding in CsCl gradient. N–RNA template was finally dialyzed against Tris-EDTA. The purity of N–RNA template was determined initially by silver staining of gels after SDS-PAGE and finally by reconstitution of transcription in vitro with recombinant L (5,27) protein and bacterially expressed P protein.

Expression of Recombinant Baculovirus L Protein

The recombinant L protein was expressed in *Spodoptera frugiperda* cells (Sf 21) infected with recombinant baculovirus BacPAK6-L containing the L gene under the control of a polyhedrin promoter, and cytoplasmic extracts containing L activity were prepared as described in detail previously (27).

Purification of Recombinant P Protein From E. coli

Recombinant P protein cloned in pET-3a was expressed in *E. coli* and purified from the inclusion bodies using guanidine hydrochloride denaturation method as described previously (5). To obtain the soluble P protein from cytoplasmic extract, DE-3 cells were freshly transformed with the P plasmid at room temperature for 16 h. Colonies were scraped and inoculated into LB/amp and allowed to grow at 25°C up to 0.3 OD. Cells were then induced with 0.4 mM IPTG for 16 h at 25°C. Cells were suspended in a buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.0, and treated with 100 μg/ml lysozyme, 0.1%. Triton X-100, and incubated at room temperature for 15 min. $MgCl_2$ was added up to 10 mM followed by DNase treatment at room temperature for 20 min. The lysate was then centrifuged at 30,000 × *g* for 20 min. The supernatant was passed through a 0.45-μm filter and purified by nickel affinity column according to the manufacturer's protocol (Novagen). Proteins were subjected to 10% SDS-

polyacrylamide gel electrophoresis as described by Laemmli (24).

GTP Binding Assay

Reaction mixtures (25 µl) containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, [α-^{32}P]GTP (20 µCi, 3000 Ci/mmol), and bacterially expressed P protein (2 µg) were incubated for 1 h at 30°C. After 1 h one tube received calf intestinal alkaline phosphatase (IU, Boehinger) for 30 min at 37°C. GTP binding of protein was analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS, followed by autoradiography.

Peptide Mapping

[α-^{32}P]GTP-labeled P protein was subjected to protease digestion. For enzymatic proteolysis of the labeled P protein, conditions that ensured complete digestion were first established by using different protein/enzyme ratios. Edolys-C digestion was carried out in a buffer containing 25 mM Tris-HCl, pH 8.5, 1 mM EDTA for 18 h at 37°C while chymotrypsin digestion was performed in a buffer containing 100 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$ for 18 h at 25°C as detailed previously (10). Endolys-C and chymotrypsin were purchased from Boehringer Mannheim Inc. The cleaved products were analyzed in 20% SDS-polyacrylamide gel (24) or in 40% alkaline-polyacrylamide gel (11), depending upon the size of the peptide.

RESULTS

Binding of GTP to Bacterially Expressed P Protein of VSV

We have shown previously that the P protein isolated from purified VSV as well as from RNP is a GTP binding protein (13). To pursue the GTP binding property of VSV P protein in detail, bacterially expressed P protein was incubated with [α-^{32}P]GTP and analyzed by electrophoresis in 10% polyacrylamide gel followed by autoradiography. Surprisingly, the bacterially expressed P protein (PO) did not bind to GTP, as shown in Figure 1. However, when PO was phosphorylated by CKII in vitro, the phosphorylated P1 form could effectively bind to GTP (Fig. 1), indicating that phosphorylation of P is required to bind to GTP. The radioactivity in GTP remained bound to the P protein even after boiling in the presence of SDS (2%) and 2-mercaptoethanol (5%), indicating covalent nature of the binding of GTP to the P protein. In a separate series of experiments (Fig. 2) we observed that under the same condition, covalent

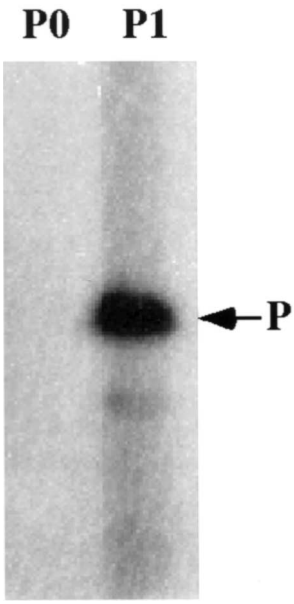

Figure 1. Binding of [α-^{32}P]GTP to VSV P protein. Bacterially expressed P protein (PO, lane 1) and P1 (obtained by in vitro phosphorylation of PO with recombinant CKII) were incubated with [α-^{32}P]GTP as described in Materials and Methods. The proteins were analyzed by electrophoresis in 10% polyacrylamide gel followed by autoradiography.

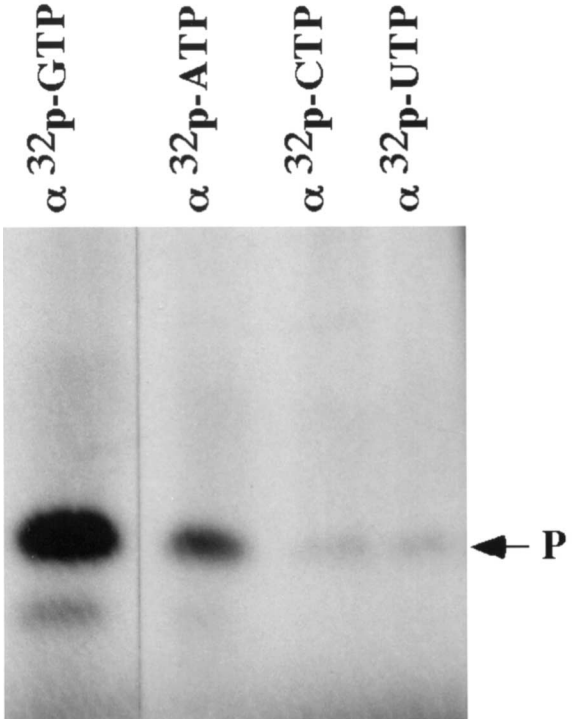

Figure 2. Binding of [α-^{32}P]NTPs to P protein. P1 (obtained by in vitro phosphorylation of PO with recombinant CKII) was incubated with 20 µCi (3000 Ci/mmol) of [α-^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]GTP, or [α-^{32}P]UTP for 1 h at 30°C. NTP binding of protein was analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS, followed by autoradiography.

binding of labeled ATP, CTP, and UTP to P1 was 15%, 5%, and 5%, respectively, indicating preferential binding of P1 to GTP.

Nature of GTP Binding to P Protein

We wanted first to determine if binding of P1 form to [α-^{32}P]GTP was mediated by covalent linkage similar to vaccinia virus or cellular guanylyltransferase (30) (i.e., E–GMP complex). As shown in Figure 3, when GTP-bound P1 protein was treated with calf intestinal alkaline phosphatase, the radioactivity in P protein was virtually eliminated. Thus, unlike the guanylyltransferase, which retains the radioactivity following phosphatase treatment, the phosphate group in P1–GTP complex is exposed and sensitive to alkaline phosphatase treatment. The band above the P protein in Figure 3 is CIAP, which was also labeled by GTP during the reaction. These results suggest that the linkage of GTP with the P protein is possibly mediated via the guanosine base and not through a phosphoamide linkage seen in the E–GMP complex (30).

Next, we carried out competition experiments with unlabeled nucleotides to determine the specificity of this observed binding with GTP. As shown in Figure 4, binding of [α-^{32}P]GTP was completely abrogated in the presence of 1 mM ATP or ADP, whereas addition of 1 mM AMP had no effect. Similarly, addition of 1 mM GTP or GDP competed out the entire radioactivity, but GMP had no effect (Fig. 4). Unlabeled CTP and UTP also had no effect (Fig. 4). Thus, it seems that ATP, ADP, GTP, and GDP can compete for the binding site(s) of GTP but not AMP, GMP, CTP, or UTP. We then wanted to determine if the GTP, once bound to P protein, could be subsequently chased with unlabeled GTP. After incubation of P1 with [α-^{32}P]GTP for 1 h, 1 mM unlabeled GTP was added and incubated at 30°C for 2 h. As shown in Figure 5, [α-^{32}P]GTP remained bound to the P1 protein following a chase with GTP, indicating that the bound GTP could not be removed by the addition of unlabeled GTP. To test whether the bound GTP to P1 could be transferred to VSV mRNA to cap the 5′ end of nascent VSV mRNA in vitro, we carried out in vitro transcription reconstitution experiment, incubating the GTP-bound P protein with N–RNA, recombinant L expressed in Sf 21 cells and NTPs for 2 h at 30°C. As shown in Figure 5, the GTP remained completely bound to P protein and was not transferred to the mRNAs during the reaction, strongly suggesting that covalent binding of GTP with the P protein is irreversible.

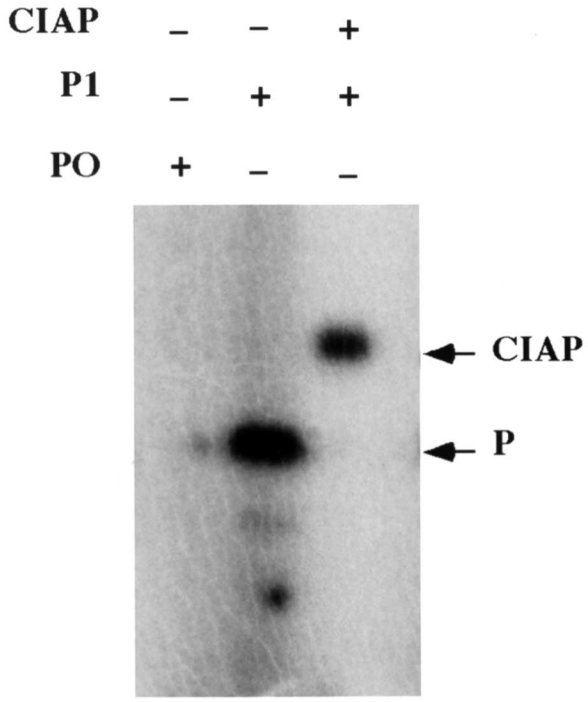

Figure 3. Effect of calf intestinal alkaline phosphatase on P–GTP complex. P1 was incubated with [α-^{32}P]GTP as described in Materials and Methods. After 1-h incubation with [α-^{32}P]GTP one tube received calf intestinal alkaline phosphastase (30 min at 37°C with IU of CIAP). The proteins were analyzed by electrophoresis in 10% polyacrylamide gel followed by autoradiography. Calf intestinal phosphatase labeled during the reaction is indicated by arrow.

Mapping of GTP Binding Site

To map the GTP binding site within the P protein we carried out the peptide mapping experiments using endolysine and chymotrypsin as detailed previously (10). The [α-^{32}P]GTP-bound P1 protein was purified from gel and digested with endolys-C. The GTP-bound peptide migrated as 75 aa in 20% SDS-PAGE as judged by the migration position of the molecular weight marker (10 kDa) (Fig. 6). The 75-aa peptide spans amino acids 35–110 in domain I of P protein, as previously reported (10). Further digestion of the 75-mer peptide with chymotrypsin produced a small peptide migrating with the bromophenol blue marker (20 aa), which spans the CKII-mediated phosphorylation site of the P protein (55–76 aa), as shown previously (10) (Fig. 6). Thus, it seems that the binding of GTP to P protein occurs within the domain where the phosphorylation occurs. However, the exact amino acid that establishes the link with GTP remains to be determined.

GTP Binding Ability of P Mutants

Finally, because bacterially expressed unphosphorylated P protein failed to bind to GTP, we wanted

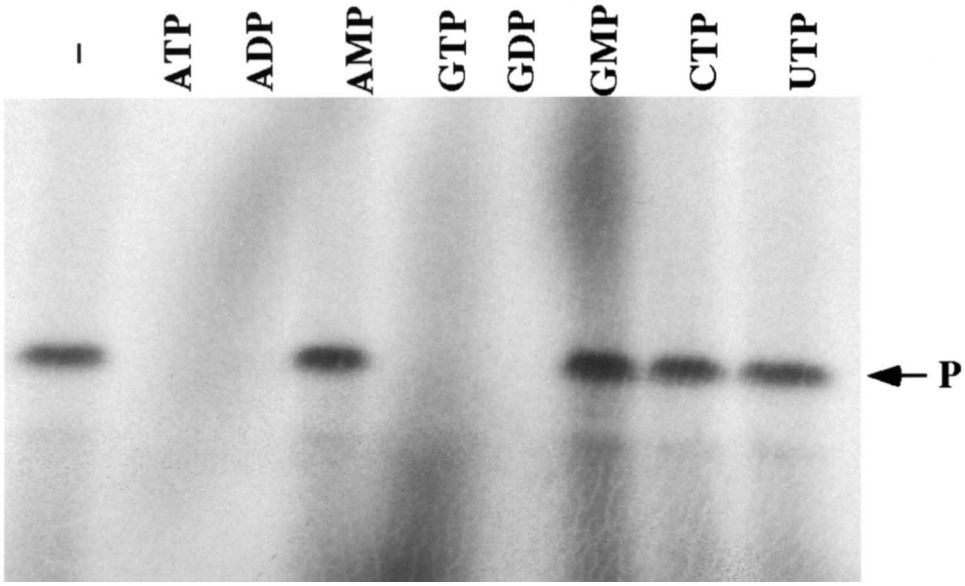

Figure 4. Competition with other nucleotides. P1 was incubated with [α-^{32}P]GTP alone or in the presence of 1 mM of unlabeled ATP, ADP, AMP, GTP, GDP, GMP, CTP, or UTP for 1 h at 30°C. The proteins were analyzed by electrophoresis in 10% polyacrylamide gel followed by autoradiography.

to determine if phosphorylation negative P mutants could possibly bind to GTP. The alanine substituted P mutants P3A (P60A, P62A, P64A), glutamic acid substituted mutant P3E (P60E, P62E, P64E), and arginine substituted mutant P3R (P60R, P62R, P64R) were used in the binding reaction. As shown in Figure 7, phosphorylation negative mutants P3A, P3E, and P3R failed to bind to GTP, indicating that phosphorylation by CKII is indeed essential for binding to GTP.

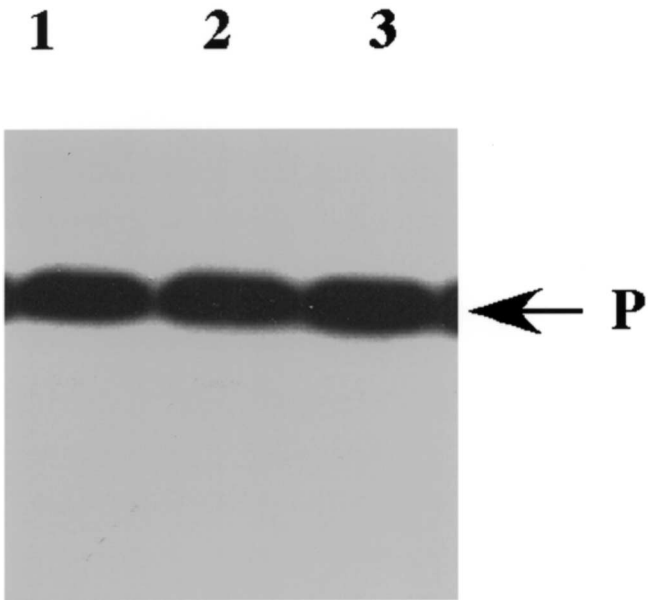

Figure 5. [α-^{32}P]GTP-bound P protein chased by cold GTP and VSV transcriptional components. P1 was incubated with [α-^{32}P]GTP for 1 h at 30°C as described in Materials and Methods. The unincorporated nucleotides were removed by passing through Nick Spin column (Sephadex G-50). GTP-bound P protein was then incubated alone (lane 1), with 1 mM cold GTP (lane 2), or with N–RNA template, recombinant L, and 1 mM of ATP, CTP, UTP, and GTP (lane 3) for 2 h at 30°C.

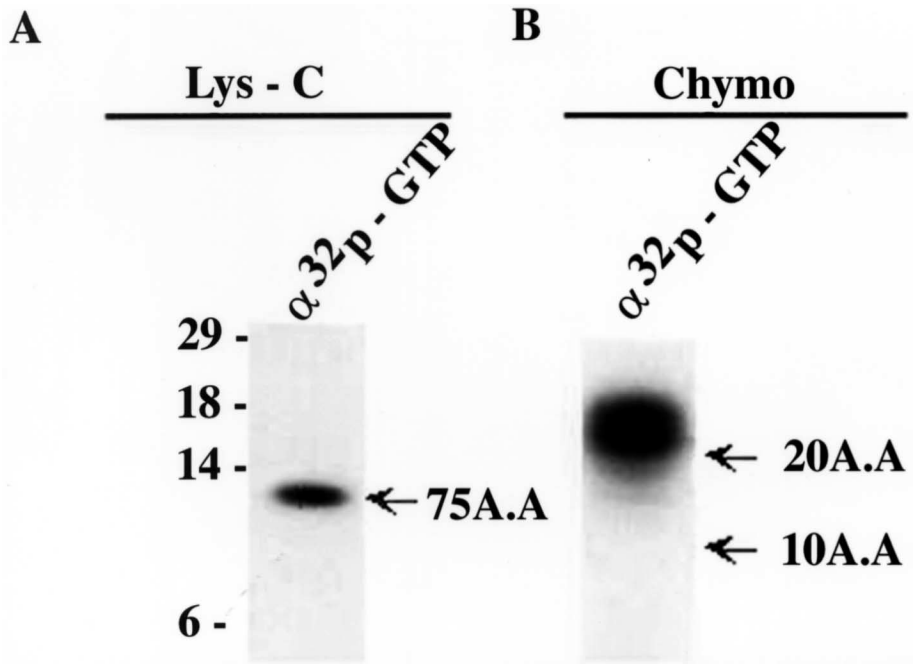

Figure 6. Peptide mapping of GTP binding to P protein. The [α-^{32}P]GTP-bound P protein was subjected to endolys-C digestion (A). The cleaved products were analyzed in 20% polyacrylamide gel followed by autoradiography. The migration position of the BRL low molecular weight markers (kDa) are shown. The 75-amino-acid-long GTP-bound peptide spanning 35–100 amino acids was further digested with chymotrypsin (B). The cleaved product spanning 55–76 amino acids was analyzed in 40% alkaline polyacrylamide gel followed by autoradiography. The migration positions of bromophenol blue (20 aa) and CKII peptide (10 aa) are shown.

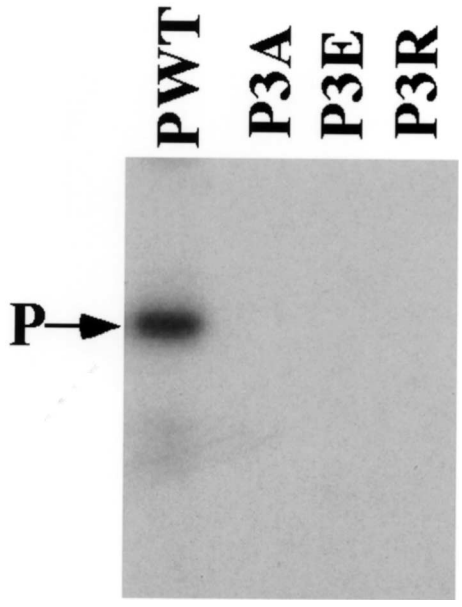

Figure 7. [α-^{32}P]GTP binding to P mutants. P1 (obtained by in vitro phosphorylation of PO with recombinant CKII), P3A, P3E, and P3R were incubated with [α-^{32}P]GTP as described in Materials and Methods. The proteins were analyzed by electrophoresis in 10% polyacrylamide gel followed by autoradiography.

DISCUSSION

In this article we demonstrate that VSV phosphoprotein P, an auxiliary protein to the RNA polymerase (L) protein, is a GTP binding protein, which preferentially binds with GTP only in its phosphorylated state (Fig. 1). The GTP remains tightly bound to P even when boiled in the presence of SDS (2%) and 2-mercaptoethanol (5%) and subjected to electrophoresis in SDS-polyacrylamide gel, strongly suggesting that GTP is covalently bound to the P protein. The covalent binding of GTP to P protein of VSV seems to be similar to the novel NTP binding property of Rice dwarf phytoreovirus (RDV) minor core protein P5, which covalently binds to GTP and was thought to be a candidate for guanylyltransferase (31). P5 protein of RDV lacks the consensus KXDG motif commonly found in other GTs and also differs from other GTs in binding not only to GTP, dGTP but also to ATP and UTP. The authors suggested that it could be a unique form of GT involved in RDV mRNA capping. However, in the VSV case, the P protein is not the GT; thus, binding of GTP may have other ramifications in its biological activity. This covalent binding of GTP to the P protein is unlike the GTP binding proteins (G-proteins) like EF-Tu, P21, etc., where GTP binds noncovalently to the proteins and

is sensitive to SDS treatment (7,23,29). Moreover, unlike E–GMP covalent complex formed by the GT (30), the P–GTP complex is sensitive to phosphatase (Fig. 3), indicating that the bond between GTP and the protein is probably via the guanosine base.

It is interesting to note that GTP binding can be effectively competed by ATP and ADP (Fig. 4), suggesting that both nucleotides may share the common binding site(s). However, ATP by itself does not bind efficiently to the P protein (about 15%) (Fig. 2), suggesting that the affinity for ATP to P protein may be considerably less than GTP. Another unique feature of GTP binding to the P protein is that once [α-^{32}P]GTP was bound to the P protein, the radioactivity could not be chased out by the addition of unlabeled GTP (Fig. 5). Furthermore, this label could not be removed even during the transcription reaction condition, suggesting that GTP may provide some unknown function to the P protein. Because unphosphorylated P, which fails to bind to GTP, is biologically active following phosphorylation, it is tempting to speculate that perhaps GTP binding plays a structural role for the P protein and imparts the optimal, biologically active confirmation. It is further supported by the observation that phosphate-defective P mutants remain inactive due to lack of phosphorylation, thus failing to bind to GTP (Fig. 7). It was previously shown that phosphorylation of P protein renders P protein to undergo multimerization. Thus, binding of GTP may further modify the structure of P protein in the presence of GTP (during transcription), the nature of which remains to be determined. It is interesting to note that in some cellular proteins, prior phosphorylation of one domain has been shown to be essential for binding to nucleotides. For example, in the case of cystic fibrosis transmembrane conductance regulator (CFTR), which is involved in CL channel activity (9), a novel regulatory mechanisms has been attributed to the cytoplasmic R domain and two cytoplasmic nucleotide binding domains (NBDs). Cytoplasmic R domain is phosphorylated by cAMP-dependent protein kinase (PKA) or protein kinase C. Once the R domain is phosphorylated, ATP interacts with the nucleotide binding domain to regulate channel activity (9). Thus, binding of ATP is contingent upon prior phosphorylation of the cytoplasmic domain R. It is also noteworthy that *E. coli* enzymes involved in glutamine synthesis are regulated by covalent modification with ATP and UTP (8). Thus, it is possible that binding of GTP to the phosphorylated P protein potentiates an important functional property that is responsible for imparting biological activity to the P protein. Current work is directed towards understanding the precise role of GTP in the P function.

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