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SPECIFIC BINDING OF GUANOSINE 5'-DIPHOSPHATE WITH THE NS PROTEIN OF VESICULAR STOMATITIS VIRUS

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A soluble protein fraction containing L, NS, G and M proteins of vesicular stomatitis virus was prepared by treatment of Triton-disrupted virions with 0.8M NaCl. Incubation of the soluble fraction with β-^{32}P GDP followed by analysis of the proteins by polyacrylamide gel electrophoresis showed specific labeling of the NS protein. The NS-GDP complex was sensitive to phosphatase treatment, suggesting non-covalent binding. No binding of GDP to NS protein was detected when the soluble fraction was pre-heated at 100°C for 1 min. or Mg^{++} was omitted from the incubation mixture. The binding was inhibited by ATP consistent with competition for a common nucleotide binding site.

In addition to the virion-associated RNA polymerase, purified vesicular stomatitis virus (VSV) contains several mRNA modification enzymes, including a guanylyltransferase that uses GTP to form a cap structure at the 5' end of the mRNA (1). In the case of VSV mRNA the cap contains the α and β phosphates of GTP and the α-phosphate of the initiating A residue of the mRNA. In addition, VSV has two methyltransferases that catalyse the transfer of methyl groups from S-adenosylmethionine to position 7 of the capping guanosine residue and to 2'-O-ribose moiety of the penultimate adenosine (2,3) and a poly(A)-polymerase that adds approximately 200 A residues to the 3'-end of the completed mRNAs (4,5). In contrast to the well-characterized reovirus and vaccinia virus capping systems, the 5'-end modifications of VSV mRNA appear to be transcription dependent (6), i.e. only nascent mRNA chains are modified. Moreover, the origin of the three phosphates in the VSV mRNA cap structure is different from the other two virus systems where only the α-phosphate of GTP is retained in the cap structure (7). Recently, it has been shown that guanylyltransferase purified from vaccinia virus forms an

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intermediate enzyme-GMP complex which transfers the GMP moiety to the 5'-end of mRNA to form the cap structure (8). The formation of the intermediate has also been shown for soluble guanylyltransferases purified from Hela cells (9,10) and rat liver (11).

Inability to cap exogenous RNA by purified VSV makes it difficult to study and determine the precise steps involved in the capping reactions. Consequently we have approached this problem by studying the ability of viral proteins to bind to GTP similar to the vaccinia virus system (8). The possible utilization of the complex, if formed, could then be studied for analyzing cap formation *in vitro*. In this communication, we report that the NS protein of VSV preferentially binds GDP. Although we have been unable to demonstrate cap formation *in vitro*, the formation of a specific NS-GDP complex indicates that this complex may have a function(s) in the life-cycle of VSV.

MATERIALS AND METHODS

Purification of VSV. VSV (Indiana serotype) was grown in baby hamster kidney cells (BHK-21, clone 13) adapted to suspension culture as described previously (12).

Solubilization of viral proteins from purified virus: Purified virus (500 µg/ml) was disrupted in a buffer containing 10mM Tris-HCl, pH 8.0, 5% glycerol, 0.8M NaCl, 1.85% Triton X-100, and 0.6 mM dithiothreitol (DTT) (13). The ribonucleoprotein (RNP) was sedimented through 30% glycerol by centrifugation in the SW60 rotor at 45,000 rpm for 2 hr. The soluble protein fraction containing L, NS, G, M and a trace of N protein remaining at the top of the centrifuge tube was dialyzed against 10mM Tris-HCl, pH 8.0, containing 20% glycerol, 0.2M NaCl, and 1mM DTT and concentrated on dry Sephadex G-200 beads (13).

GDP binding assay: The reaction mixture (0.05ml) contained in an Eppendorf tube 50mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10mM DTT, 1 µM β-^{32}P GDP (40Ci/mmole) or α-^{32}P GTP (410 Ci/mmole), 20 µg of purified virus and 0.05% Triton N-101. When soluble protein fraction (15 µg) was used, Triton N-101 was omitted. The incubation was carried out at 30°C for 20 min, and the reaction was terminated by adding 6 µl 0.1M EDTA and 75 µl ice-cold 10% trichloroacetic acid (TCA). The acid-insoluble material recovered by centrifugation was suspended in cold 5% TCA and recentrifuged. The pellet was washed with ether, dried and dissolved in gel sample buffer containing 1% 2-mercaptoethanol. Samples were heated at 100°C for 4 min and analyzed by electrophoresis on 10% polyacrylamide gel containing 1% SDS (14). The gels were autoradiographed using Kodak XR5 films either directly or after vacuum drying.

Assay conditions for α-^{32}P GTP binding to vaccinia virus guanylyltransferase were as described by Schuman and Hurwitz (8).

Preparation of β-^{32}P GDP: β-^{32}P GDP was prepared enzymatically using γ-^{32}P ATP and GMP in the presence of GMP-kinase according to the method of Furuichi and Shatkin (15). The labeled GDP was purified by chromatography on PEI-cellulose paper as described earlier (16).

Materials: (α-^{32}P)-ribonucleoside triphosphates were purchased from Amersham. Calf intestinal alkaline phosphatase was from Boehringer, Mannheim. Purified vaccinia virus was a gift from Drs. Y. Furuichi and D. Wang.

RESULTS

To determine if any of the VSV structural proteins possess the ability to form a GMP complex similar to vaccinia virus, Triton-disrupted purified VSV was incubated with α-^{32}P GTP. The product was precipitated with trichloroacetic acid, heated with sodium dodecylsulfate (SDS) containing 2-mercaptoethanol and analyzed by electrophoresis in an SDS-polyacrylamide gel. As shown in Fig. 1 (lane A), only the NS and the M protein of VSV contained significant radioactivity. The incorporation of radioactivity in the two proteins was approximately 1:1. Since the mass ratio of NS to M in the virus particle is approximately 1:6, the relative activity of α-^{32}P GTP bound to NS was 6-fold higher than the M protein. Consistent with this value, NS and M proteins contained 1520 cpm/µg and 200 cpm/µg, respectively as

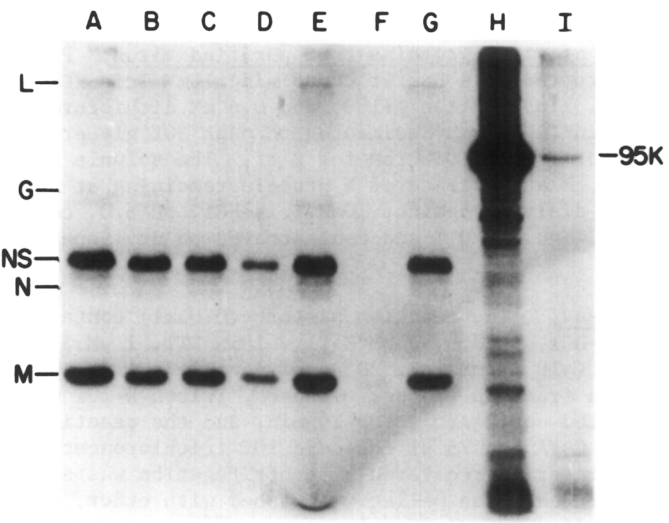

Fig. 1. Binding of α-^{32}P GTP with VSV Structural Proteins: Triton-disrupted purified VSV was incubated with α-32P GTP alone (A) or in the presence of unlabeled GTP (100 µm) (B), GTP (1mM) (C), GDP (1mM) (D), GMP (1mM) (E), ATP (1mM) (F), ITP, (1mM) (G). The proteins were subsequently processed and analyzed by electrophoresis in 10% polyacrylamide gel followed by autoradiography as described in Materials and Methods. Detergent disrupted purified vaccinia virus (20 µg) was incubated with α-^{32}P GTP (H) and in the presence of unlabeled GTP (1mM) (I) according to the method of Schuman and Hurwitz (8) and processed as above. Migration positions of VSV structural proteins are shown. The 95K protein represents vaccinia guanylyltransferase - GMP complex (8).

determined from Fig. 1. These results indicated that the binding observed was more specific for NS than M protein. Surprising results were obtained when isotopic dilution experiments were performed with unlabeled GTP. As shown in Fig. 1, Lane B and C, dilution of α-^{32}P GTP with 100 μM and 1mM unlabeled GTP, respectively, reduced the bound radioactivity by only 20%. By contrast, addition of GDP (1mM) reduced the protein-associated radioactivity by more than 90% (Lane D). GMP (1mM) and ITP (1mM) had no effect on the radioactivity bound to NS and M proteins shown in lanes E and G respectively. However, unlabeled ATP (1mM) virtually removed the entire radioactivity (lane F). In control experiments using purified vaccinia virus, it was found that a 95,000 dalton protein was associated with α-^{32}P GTP (8) (Lane H). This label, as expected, was reduced by more than 95% by the addition of 1mM unlabeled GTP (Lane I). Thus, it seemed that the α-^{32}P GTP complex with NS and M protein of VSV contained α-^{32}P GDP since isotopic dilution occurred in the presence of GDP. Moreover, ATP had an inhibitory effect on the binding of α-^{32}P GDP suggesting competition for a common binding site.

We next prepared β-^{32}P GDP and incubated with Triton-disrupted VSV. As shown in Figure 2 (lane A), the NS and the M proteins of VSV were predominantly labeled. The relative amount of β-^{32}P GDP bound to the NS protein was 10-fold higher than observed with α-^{32}P GTP (Fig. 1). A low level of incorporation of α-^{32}P GDP also occurred with other viral structural proteins. The radioactive bands migrating between NS and M proteins were not identified. The β-^{32}P GDP was decreased by more than 90% when the reaction mixture was diluted with unlabeled GDP (1mM) (Lane B). Thus, it seemed that GDP and not GTP formed complexes with the NS and M proteins of VSV. In a separate series of experiments it was found that α-^{32}P CTP and α-^{32}P UTP were not complexed with the NS protein. However, a low level of binding (10% with respect to α-^{32}P GTP) was observed with α-^{32}P ATP.

Since the structural proteins G, M, NS, and L of VSV can be removed from the N-protein RNA complex by treatment with salt (17), it was of interest to test whether the same two proteins in the soluble protein pool namely

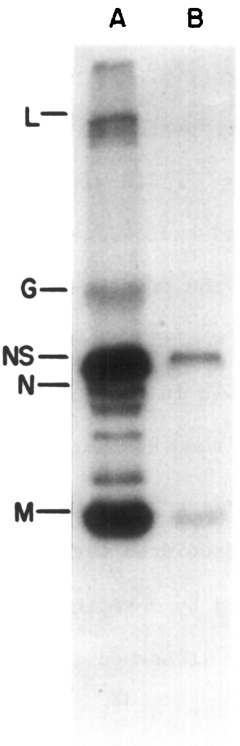

Fig. 2. β-^{32}P GDP binding to VSV structural proteins. Triton-disrupted VSV was incubated with β-^{32}P GDP (A) or in the presence of GDP (1mM) (B) as described in Materials and Methods. The reaction products were processed as described in Figure 1. Migration positions of VSV structural proteins are shown.

NS and M, are able to bind GDP. The salt extracted protein mixture was incubated with β-^{32}P GDP and analyzed by SDS-polyacrylamide gel electrophoresis. In contrast to the Triton-disrupted virus, the predominantly labeled protein in the gel was the NS protein (Fig. 3, lane D). NS protein contained 20-fold greater radioactivity than the M protein. Again, some faint, unidentified bands were seen migrating between NS and M. These results indicate that the specificity of GDP binding to the NS protein was more pronounced after the proteins were solubilized from the virion.

Various parameters for GDP-binding to the NS protein were studied using the soluble protein fraction as the source of the NS protein. The binding was totally dependent on Mg^{++}, since no labeling of the NS protein occurred when the divalent cation was omitted from the reaction mixture (Fig. 3, Lane B). Also, heated protein fraction failed to label the NS protein (Lane A). Optimum

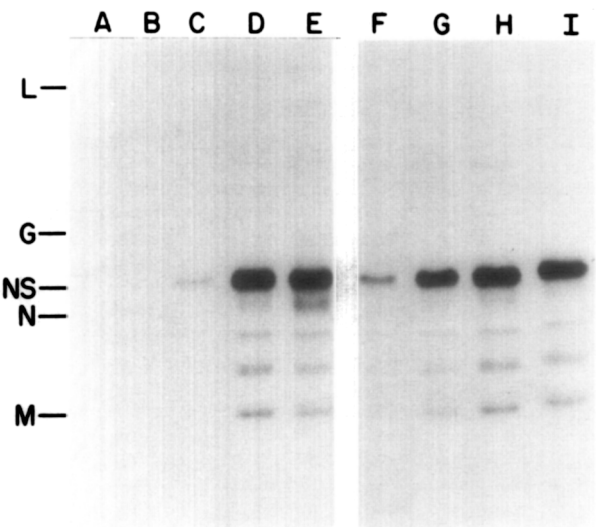

Fig. 3. Requirements for β-^{32}P binding to NS protein. Solubilized viral structural proteins were incubated with β-^{32}P GDP and the proteins analyzed as described in Materials and Methods. Following are the reaction conditions: (A) heated (100°C, 1 min.) protein fraction was used in the reaction; (B) Mg^{++} was omitted; (C) incubated at 4°C, (D) complete reaction; (E) Mg^{++} was replaced by Mn^{++} (1.5mM); (F), (G), (H), (I), represent binding at 5, 10, 20, 30 min. incubation period, respectively. Migration positions of VSV structural proteins are shown.

labeling of the NS protein occurred at 30°C (Lane D), although low (less than 5%) but reproducible labeling occurred at 4°C (lane C). Mn^{++} (1.5mM) efficiently replaced Mg^{++} (Lane E). Maximum labeling of NS protein occurred within 20 min. of incubation at 30°C (Lanes F to I). From these results it seemed that binding of β-^{32}P GDP to the NS protein was mediated by an enzyme.

If indeed the binding of NS protein to β-^{32}P GDP was mediated by covalent linkage similar to the vaccinia virus guanylyltransferase (8), the labeled phosphate in GDP would be resistant to the treatment of calf intestinal alkaline phosphatase. As shown in Figure 4, when similar experiments were performed with NS-GDP complex, the NS-GDP complex lost virtually all radioactivity when treated with phosphatase (Lane B). As expected, the 95K protein-GMP complex of vaccinia virus retained virtually the entire radioactivity following phosphatase treatment (Lane D). The labeled band (shown by an arrow) was calf intestinal alkaline phosphatase which was labeled by β-^{32}P GDP or GTP during the reaction. Thus, it appeared that the NS-GDP complex was not a

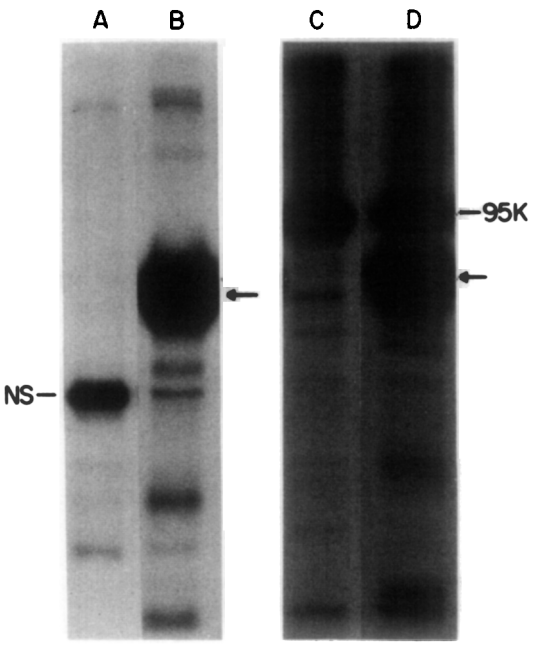

Fig. 4. Effect of calf intestinal alkaline phosphatase on NS-GDP complex. Soluble VSV proteins or solubilized vaccinia virus were incubated with β-^{32}P GDP and α-^{32}P GTP, respectively, as described in Materials and Methods. After 30 min. one tube received calf intestinal alkaline phosphatase (1 hr at 37°C with 50 U/ml). The proteins were processed and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. VSV, without (A) and with (B) phosphatase; Vaccinia virus without (C) and with (D) phosphatase. The 95K vaccinia guanylyltransferase is shown. The arrows represent calf intestinal alkaline phosphatase labeled during reaction.

covalent complex of the form NS-$\overset{*}{p}$pG, rather possibly a tight association with the GDP or covalent linkage of the type NS-G$\overset{*}{p}\overset{*}{p}$.

As shown in Figure 5, once β-^{32}P GDP was bound to the NS protein, the GDP moiety could not be removed by the addition of GDP (Lane B), GTP (lane C), ATP (Lane D), P_i (Lane E), and PP_i (Lane F). Moreover, the NS-GDP complex was resistant to acid hydrolysis (Lane H) but totally sensitive to alkali treatment (Lane I). These properties of the complex are quite different from the vaccinia virus system where formation of the enzyme-GMP complex was inhibited by PP_i and the complex was sensitive to acid and not alkali (9). Attempts were made to transfer GDP from NS-GDP complex to guanylylate the 5'-end of oligonucleotides such as pppApG, ppApG, or nascent VSV mRNAs *in vitro*. No label from the complex could be transferred to any of these potential acceptor species to form caps (data not shown).

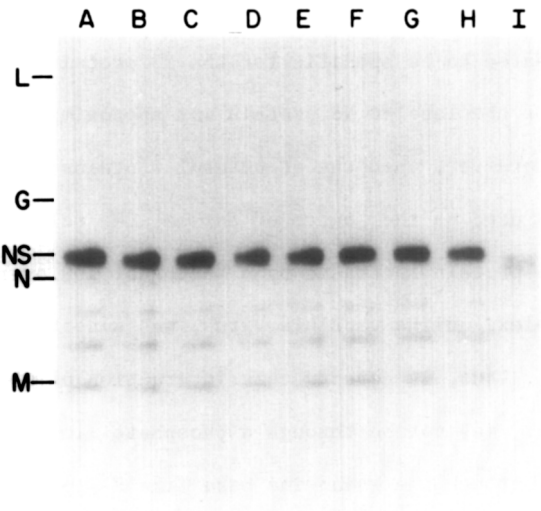

Fig. 5. Properties of NS-GDP Complex. Soluble VSV structural proteins were incubated in separate tubes in the presence of β-^{32}P GDP as described in Materials and Methods. After 30 min of incubation the following additions were made and the reaction continued for 30 min. The proteins were subsequently analyzed by polyacrylamide gel electrophoresis. (A), no addition; (B) 1mM GDP; (C) 1mM GTP; (D) 1mM ATP; (E) 1mM Pi; (F) 1mM PPi.

In a separate set of reactions in the presence of β-^{32}P GDP, the proteins were precipitated with cold 5% TCA and the pellet washed with ether and suspended in water. (G) The suspension was heated at 70°C for 10 min., (H) the suspension was made to 0.1N HCl and heated at 70°C for 10 min., (I) the suspension was made to 0.1N NaOH and heated at 70°C for 10 min. The migration positions of VSV structural positions are shown.

DISCUSSION

From the detailed studies in the vaccinia virus and cellular systems, it has been shown that the enzyme that guanylylates 5'-termini of mRNA forms a stable enzyme - pG covalent intermediate when incubated with α-^{32}P GTP (8-11). Using this approach, we have attempted to identify and characterize the polypeptide in purified VSV that catalyzes similar guanylylation of the nascent mRNAs in vitro. Incubation of Triton-disrupted virions with α-^{32}P GTP resulted in labeling of both NS and M proteins (Fig. 1). Isotopic dilution experiments revealed that the label incorporated was α-^{32}P GDP that was contaminating the labeled ribonucleoside triphosphate. Using purified β-^{32}P GDP it was shown that the labeling of NS and M proteins was increased 10-fold indicating that the labeling was indeed mediated by GDP (Fig. 2). This observation was consistent with the finding that the α and β

phosphates of GTP are retained in the VSV mRNA cap structure (1). The binding of GDP appeared to be specific for the NS protein because the specific activity of the labeled NS protein was approximately 6-fold higher than M protein. Moreover, when the structural proteins were solubilized from the virion and used as the source of enzyme, the relative activity of the label in the NS protein increased by a factor of 100 over the M protein. The GDP in the complex, unlike vaccinia virus, was sensitive to phosphatase treatment (Fig. 4). Thus, it appears that interaction of GDP with the NS protein, if covalent, may not be through a phosphate linkage. Alternatively, the linkage may be through the guanosine base like E-Gpp. This is consistent with the observation that once the NS-GDP complex has been made, the label could not be removed upon incubation with GDP (Fig. 5). Another possibility is that the interaction of GDP to NS is a non-covalent tight association, similar to the GDP-binding property of the P21 protein of Harvey Sarcoma Virus (18). However, unlike P21-GDP complex, the NS-GDP complex was resistant to SDS treatment. Thus, the precise nature of the interaction of GDP with the NS protein is presently unclear.

The interaction appears to be enzymatically mediated, since the reaction has an absolute requirement for a divalent cation and heating the protein fraction eliminated binding with GDP (Fig. 3). Complex formation, however, was not inhibited by either P_i or PP_i (Fig. 3) and the complex was sensitive to treatment with alkali. These observations again indicate that the nature of the bond between the NS protein and GDP was not a phosphoamide linkage as in other guanylyltransferase enzymes (8-10). It is interesting to note that ATP inhibited complex formation (Fig.1). This result suggests that ATP is competing for the GDP-binding sites.

NS is a phosphoprotein (19) which has been shown to be an essential component involved in the transcription process of the viral genome in vitro (17). Although the present data indicates that the NS-GDP complex may not be involved in the capping reaction, the complex may have other regulatory

function(s) in some steps of the virus replicative cycle. Further experiments are needed to determine the function of this complex.

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