Evidence for Guanylate Cyclase Activity Associated with Hemagglutinating Virus of Japan (Sendai Virus)*

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Purified virions of HVJ (Sendai virus) were found to contain a guanylate cyclase activity that converts GTP to cyclic GMP. Activities of adenylate cyclase and 5'-nucleotidase which are frequently used as marker enzymes of cell membranes were not detected in the virus. Guanylate cyclase and virion-associated activities, neuraminidase and hemagglutinin, were co-purified during a purification of virions. Guanylate cyclase activity was not detected without disruption of the virions with a detergent, Triton X-100 or Nonidet P-40. Treatment of intact HVJ with a proteolytic enzyme, trypsin or chymotrypsin, destroyed both neuraminidase and hemagglutinin; however, most of the guanylate cyclase was retained. Guanylate cyclase activity was found in fractions containing nucleocapsids after sucrose gradient centrifugation of disrupted virions. These results indicated that the enzyme was tightly bound to cores of HVJ and, therefore, its presence could not be explained by binding of host cell enzyme to the surface of virions. Properties of the virus-derived enzyme and particulate fractions of host cell homogenates were similar. Antiserum against nucleocapsids of HVJ inhibited guanylate cyclase activity of HVJ and particulate fractions of cells such as chorioallantoic membrane and rat liver, while soluble guanylate cyclase was not inhibited by antiserum. The biological significance and origin of guanylate cyclase found in HVJ are obscure and await further study.

Guanylate cyclase, which catalyzes the formation of cyclic GMP from GTP, has been detected in all organisms so far examined (1). However, the presence of guanylate cyclase in viruses has not been reported. In this paper, we describe a tightly bound guanylate cyclase activity in cores of HVJ. We were unable to detect activities of adenylate cyclase and 5'-nucleotidase, used as marker enzymes of cell membrane, in highly purified HVJ. Although the origin and function of guanylate cyclase found in HVJ are not clear, our findings are of interest in view of the presence of protein kinase in viruses (2-7). Our observations suggest an involvement of cyclic GMP in the interaction between host cell and virus.

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† The abbreviation used is: HVJ, hemagglutinating virus of Japan.

MATERIALS AND METHODS

RESULTS

Purification of HVJ and Guanylate Cyclase Activity—Guanylate cyclase and virion-associated activities, neuraminidase and hemagglutinin, were determined during purification of HVJ. As shown in Table I, recoveries of 3 activities were parallel during purification of HVJ from chorioallantoic fluid. Although the specific activity of neuraminidase and guanylate cyclase decreased slightly in this experiment, those of both enzymes in other experiments showed no change or increased slightly and the parallel purification of both enzymes was obtained in all experiments. Guanylate cyclase activity sedimented with virions in the second linear sucrose density gradient centrifugation (Fig. 2 in miniprint), and activities of neuraminidase and hemagglutinin of HVJ were also found in this peak. Electron micrography of pooled samples (fraction 8 to 11) showed virions of HVJ, and fragments of cell structures were not detected (Fig. 3A). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified virions of HVJ showed eight identifiable polypeptides and several minor polypeptides (Fig. 4), analogous to previous publications (19-23). When the particulate fractions of homogenates of whole chick embryo (specific activity 128 pmol/min/mg) or chorioallantoic membranes (specific activity 1650 pmol/min/mg), prepared in the same manner as virions of HVJ, were subjected to centrifugation as shown in Fig. 2, a peak of guanylate cyclase was found in fractions 2 and 3 (not shown). Furthermore, the activities of adenylate cyclase and 5'-nucleotidase, which are frequently used as marker enzymes of cell membranes, were not detected in the purified preparations of HVJ (Table I). These results indicated that guanylate cyclase activity found in preparations of HVJ was not due to contaminating fragments of cell membranes.

Effect of Proteolytic Enzymes—When virions of HVJ and influenza virus are treated with proteolytic enzymes, the surface glycoproteins are removed without alteration of the internal proteins (20-23). Therefore, to exclude a possible binding of guanylate cyclase of host cell on the surface of the virion, virions of HVJ were treated with trypsin or chymotrypsin (Fig. 5). Treatment with trypsin or chymotrypsin at a
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Purification of HVJ and marker activities for the virion and the plasma membrane

Crude HVJ preparation was obtained by centrifugation of chorioallantoic fluid at 35,000 x g for 30 min. Further purification of HVJ and determination of enzymatic activities are described under "Materials and Methods" in the miniprint supplement.

Table I

| Fractions       | Protein (mg) | Hemagglutinin (%) | Neuraminidase | Guanylate cyclase | Adenylate cyclase and 5'-nucleotidase |
|-----------------|--------------|-------------------|---------------|-------------------|--------------------------------------|
|                 |              |                   | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity |
| Crude HVJ       | 39.7 x 10^{-3} | 251               | 1.69 µmol/min/mg  | 67.1 µmol/min  | 42.0 µmol/min/mg | 1.67 µmol/min  | 100 µmol/min/mg  | 100 µmol/min/mg |
| First sucrose   | 26.4         | 128               | 1.22 µmol/min/mg  | 32.2 µmol/min  | 34.8 µmol/min/mg | 0.92 µmol/min  | 65 µmol/min/mg   | Not detectable  |
| Second sucrose  | 21.5         | 95                | 1.69 µmol/min/mg  | 23.5 µmol/min  | 32.1 µmol/min/mg | 0.69 µmol/min  | 41 µmol/min/mg   | Not detectable  |

The limits of detectable activity are 0.02 pmol/min/mg for adenylate cyclase and 0.5 nmol/min/mg for 5'-nucleotidase with 0.5 mg of protein used/assay tube.

FIG. 3. Electron micrographs of HVJ and components isolated from disrupted virions of HVJ. A, intact HVJ, x 7,000; B, envelopes, x 25,000; C, mixture of envelopes and nucleocapsids, x 25,000; and D, nucleocapsids, x 25,000. B–D correspond to Fractions II, IV, and VI of Fig. 7, respectively.

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified HVJ. Purified virions of HVJ (about 200 µg of protein) were boiled and subjected to electrophoresis (60 V, 6 h) as described under "Materials and Methods in the miniprint supplement.

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of HVJ treated with trypsin or chymotrypsin. 0.7 mg/ml and 3.5 mg/ml of virions of HVJ were treated at 37°C with a proteolytic enzyme at a concentration of 20 µg/ml for 60 min and 100 µg/ml for 15 min, respectively. After incubation, aliquots were added to reaction mixtures for determination of guanylate cyclase activity and incubated for 15 min. Aliquots remaining were lyophilized and subjected to electrophoresis as described under "Materials and Methods" in the miniprint supplement. A, HVJ treated with trypsin, 100 µg/ml; B, HVJ treated with chymotrypsin, 100 µg/ml; C, LMW calibration kit (Pharmacia) for standard proteins; D, untreated HVJ; E, HVJ treated with trypsin, 20 µg/ml; F, HVJ treated with chymotrypsin, 20 µg/ml. The remaining guanylate activities after the indicated treatments are given.

Concentration of 20 µg/ml for 60 min at 37°C successfully removed the surface polypeptides, HN and F. Other polypeptides which are internal appeared to remain intact. Morphologically, the spikes seen on the surface of intact virions were not observed after treatment with trypsin or chymotrypsin (not shown). Under these conditions, 77% of the guanylate cyclase activity was recovered with trypsin treatment and 91% with chymotrypsin treatment. At a concentration of 100 µg/ml, trypsin digested almost all polypeptides of virions and no guanylate cyclase activity was found, while 67% of the activity was recovered with chymotrypsin. Following chymotrypsin treatment, fragments of Mr = 40,000–43,000 were found with
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Summary of the Fractionation of disrupted virions of HVJ

Purified HVJ was treated with 0.5% Tween 20 and 1 M KCl in an alkaline solution. Aliquots (0.1 ml), immediately (zero time) and after 60 min at room temperature, were added to 0.1 ml of 0.2 M Tris-HCl buffer, pH 7.2, and stored at 4°C until activities were determined. After 60 min at room temperature, HVJ was centrifuged at 105,000 × g for 60 min. The supernatant obtained was neutralized and the pellet was suspended with alkali-Tween20-KCl. Aliquots of suspended pellet at zero time and 60 min at room temperature were neutralized and stored as above. The suspended pellet after 60 min at room temperature was subjected to centrifugation through linear sucrose density gradient. Activities of guanylate cyclase and neuraminidase were determined as described under "Materials and Methods" in the miniprint supplement.

Table II

| Fractions | Protein | Neuraminidase | Guanylate cyclase |
|-----------|---------|---------------|------------------|
|           | Specific activity (μmol/min/mg) | Total activity (μmol/min) | Percentage | Specific activity (μmol/min/mg) | Total activity (μmol/min) | Percentage |
| Disrupted virions |         |               |                  |            |                         |           |
| Zero time | 8.73    | 1.17          | 10.2             | 100        | 28.4                    | 248       | 100        |
| 60 min    | 8.73    | 1.34          | 11.7             | 100        | 15.2                    | 133       | 100        |
| Supernatant 60 min | 4.15    | 1.71          | 7.1              | 61         | 8.8                     | 37        | 28         |
| Pellet    |         |               |                  |            |                         |           |
| Zero time | 3.71    | 0.54          | 2.0              | 17         | 28.3                    | 105       | 79         |
| 60 min    | 3.71    | 0.59          | 2.2              | 19         | 16.8                    | 62        | 47         |
| Pellet 60 min | 3.71    | 0.59          | 2.19             | 100        | 16.76                   | 62.33      | 100        |
| Fraction I | 0.10    | 0.60          | 0.06             | 2.7        | 1.86                    | 0.19       | 0.3        |
| Fraction II | 0.56    | 3.30          | 1.85             | 84.5       | 3.81                    | 2.13       | 3.4        |
| Fraction III | 0.26    | 0.81          | 0.21             | 9.6        | 14.41                   | 3.75       | 6.0        |
| Fraction IV | 0.60    | 0.36          | 0.22             | 10.0       | 27.92                   | 16.75      | 26.9       |
| Fraction V | 0.45    | 0.49          | 0.22             | 10.0       | 10.79                   | 4.86       | 7.8        |
| Fraction VI | 0.33    | 0.04          | 0.02             | 0.9        | 11.22                   | 5.95       | 9.5        |

no other detectable viral polypeptides (Fig. 5). However, it is known whether or not this fragment shows guanylate cyclase activity. The finding suggests the possible location of guanylate cyclase activity within the virion.

Fractionation of Polypeptides of the Virion—Virions of HVJ, 1 mg/ml, were treated with 0.5% Tween 20 and 1 M KCl in alkaline solution to prepare the nucleocapsids as described by Hosaka (11). The results are summarized in Table II and Figs. 3, 6, and 7. When virions were treated with alkali-Tween 20-KCl, guanylate cyclase activity was quite unstable. Forty to fifty per cent of the activity was lost at room temperature after 60 min. After centrifugation of disrupted virions at 105,000 × g for 1 h, most of neuraminidase was recovered in the supernatant, while guanylate cyclase was found in the pellet (Table II). Polypeptides in the supernatant and pellet analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that all major polypeptides were found in both fractions, however, HN and F were primarily in the supernatant and P, NP, and M in the pellet (Fig. 6, lanes 2–4). Therefore, the pellet was again treated with alkali-Tween 20-KCl and subjected to centrifugation through a linear sucrose density gradient (Fig. 7 in miniprint). Three visible bands were obtained which correspond to Fractions II, IV, and VI in Fig. 7. Neuraminidase and hemagglutinin were found in the first band (Fraction I) and guanylate cyclase in the second and third bands (Fractions IV and VI) with the highest activity in Fraction IV (Table II). Polypeptide analyses revealed that envelope glycoproteins, HN and F, were present primarily in Fractions II and III (Fig. 6, lanes 6 and 7) and nucleocapsid proteins, P and NP, in Fractions IV–VI (Fig. 6, lanes 8–10). High molecular weight polypeptides and polypeptide 5 which are nucleocapsid proteins (24) and two unidentified polypeptides, M, of about 43,000, one of which was assumed to be polypeptide 7, were also present in Fractions IV–VI. Polypeptide M was seen in all fractions obtained. By electron microscopic study, free envelopes were found in Fraction II, complexes of envelopes and nucleocapsids in Fraction IV, and nucleocapsids without envelopes in Fraction VI (Fig. 3). Results of electrophoretic and electronmicroscopic studies suggested the possible association of guanylate cyclase activity with nucleocapsids of HVJ.

Discussion

In the present study, we demonstrated guanylate cyclase activity associated with purified virions of HVJ. The enzyme activity was found after disruption of virions by detergents and was resistant to limited proteolysis of intact HVJ with trypsin or chymotrypsin (Fig. 5). After disruption of virions by treatment with alkali-Tween 20 or Triton X-100 in high...
salt solution, most of the guanylate cyclase activity was recovered in the pellet upon centrifugation (Table II). Following further fractionation of the pellet by sucrose density gradient centrifugation, guanylate cyclase activity was found in fractions containing nucleocapsids (Figs. 3 and 7). These results indicate that guanylate cyclase activity is tightly associated with cores of HVJ and its presence cannot be accounted for by a simple binding of host enzyme to the surface of virions. In addition, activities of cell membrane-bound enzymes such as adenylyl cyclase and 5'-nucleotidase were not detected in the virus.

It is not clear whether guanylate cyclase in HVJ is coded by the virus genome or derived from host cells. It is known that the enveloped virus is formed at the cell surface by budding. In the mechanism of budding, it is assumed that the initial insertion of envelope proteins is followed by their migration in the cell membrane, resulting in the formation of a patch of virus-specific membrane, in which M protein plays a prominent role, guanylate cyclase activity during isolation of viral polypeptides was so similar to that of M protein of HVJ (Figs. 6 and 7) that guanylate cyclase may be located between the envelope and nucleocapsid, like M protein (22, 23).

Mammalian tissues contain two forms of guanylate cyclase, soluble and particulate forms, with different properties (1) and different antigenicity (33). The characteristics of guanylate cyclase activity of HVJ were similar to that of the particulate form of guanylate cyclase and both activities were inhibited more than 90% by antisera against nucleocapsids of HVJ (Fig. 8). The antisera showed no effect on the soluble form from avian and mammalian cells. These results indicated that the guanylate cyclase found in HVJ has properties and antigenicity similar to that of the host particulate enzyme and suggested that the enzyme in HVJ could be host cell in origin. However, several proteins from normal host cells and from viruses have been shown to have a common antigenicity (34–38). Guanylate cyclase of HVJ and membrane-bound guanylate cyclase of both chick and rat tissues might be another example of the same antigenicity between viral protein and normal cell protein.

There is no evidence, at present, that cyclic GMP which may act as a regulator of some biological processes (39) participates in viral replication, maturation, and assembly. Protein kinase activity found in various viruses is independent of cyclic nucleotides (2–7). Therefore, the role of tightly bound guanylate cyclase in HVJ is obscure at present. However, the biological functions of several enzymes found in viruses, most of which are minor polypeptide components, have become apparent by recent studies. These include: (i) protease in the maturation and assembly of virus (30, 31, 40); (ii) protein kinase that activates the reverse transcriptase activity (6, 7); and (iii) nucleoside diphosphate kinase in the synthesis of mRNA (29). Additional studies are required to test for the presence of guanylate cyclase in other viruses and to determine the biological significance of these observations.

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**Guanylate Cyclase Activity in HVJ**

Evidence for guanylate cyclase activity associated with MVN (Sendai virus) virus particles was detected by a technique described previously (13). The separation of guanylate cyclase activity was determined by the method of Solomon et al. (14). For adenylate cyclase activity, the reaction mixture was the same as those of cyclic AMP measurement, and the activity was determined by the method of Lowry et al. (17).

**Materials and Methods**

**Virus and Its Treatment**: HVJ (strain 2) was inoculated into the chorioallantoic cavity of 10-day-old chick embryos at doses of 10^2.2 plaque-forming units (PFU) per egg. Eggs were incubated at 35°C for 3 days, then stained and the chorion was removed. Cells were harvested at 1 day post-inoculation and incubated in 5% CO₂ at 35°C. The resulting supernatant was concentrated by centrifugation at 15,000 x g for 30 min and washed with distilled water containing 0.2 M Na-acetate buffer, pH 4.0. HVJ was isolated by density gradient centrifugation. Viral pellets were layered on 10 ml of 40% sucrose in HVJ and centrifuged at 30,000 rpm for 30 min. The pellets were resuspended in a small volume of PBS and layered onto 1.0 M sucrose in PBS at 27,000 rpm for 1.5 hr. The visible band of virus at the interface between 30 and 40% sucrose was collected with cell strainer and diluted with 1ml of 0.2 M Na-acetate buffer, pH 4.0. The virus solution was diluted 1:500 with 0.2 M Na-acetate buffer, pH 4.0.

**Guanylate Cyclase Activity**: The guanylate cyclase activity was determined by the method of Solomon et al. (14). For adenylate cyclase activity, the reaction mixture was the same as those of cyclic AMP measurement, and the activity was determined by the method of Lowry et al. (17).

**Antiserum**: Anti-GTPyS antiserum was obtained from rabbits immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B.

**Results**

**Formation of cyclic GMP by virus**

Formation of cyclic GMP by virus was observed when purified virus was mixed with NMN (15) (Fig. 1). The formation of cyclic GMP by virus was proportional to the inoculation time and amount of virus present in the assay. The activity was detectable in the absence of a monomer, NADg, NGF-40 or NGF-50, R (23). The cell-free reaction mixture contained 0.2 M Na-acetate, pH 4.0 and 10 mM GTPyS. A small aliquot was taken at the desired time point, heated at 70°C for 10 min, and applied to a polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue. The protein bands were visualized under UV light and photographed. The activity was calculated from the peak area of GMPase activity.

**Antiserum against GTPyS**: Anti-GTPyS antiserum was obtained from rabbits immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B. Immunized rabbits were immunized with GTPyS coupled to CNBr-activated Sepharose 4B.

**Activation of guanylate cyclase by cyclic GMP**: The activation of guanylate cyclase by cyclic GMP was detected by the method of Solomon et al. (14). For adenylate cyclase activity, the reaction mixture was the same as those of cyclic AMP measurement, and the activity was determined by the method of Lowry et al. (17).

**Effect of cyclic GMP on guanylate cyclase activity**: The effect of cyclic GMP on guanylate cyclase activity was determined by the method of Solomon et al. (14). For adenylate cyclase activity, the reaction mixture was the same as those of cyclic AMP measurement, and the activity was determined by the method of Lowry et al. (17).

**Study with anti-GTPyS antiserum**: The effect of guanylate cyclase activity on the activation of guanylate cyclase was determined by the method of Solomon et al. (14). For adenylate cyclase activity, the reaction mixture was the same as those of cyclic AMP measurement, and the activity was determined by the method of Lowry et al. (17).
Guanylate Cyclase Activity in HVJ

Characteristics of guanylate cyclase activity in HVJ. Guanylate cyclase activity from mammalian tissues requires different cations for the activity, and its requirement for Mn²⁺ is quite specific [1]. Guanylate cyclase in HVJ also had a specific requirement for Mn²⁺, but it was inhibited strongly by Mg²⁺ in excess of GTP. Mg²⁺ is a poor metal for guanylate cyclase activity in HVJ as well as in the enzyme in tissues. Galactose, at 3 mM, strongly inhibited the enzyme activity of HVJ. The apparent Kᵢ for Mg²⁺ was calculated from double reciprocal plots of enzyme activity against concentration of Mg²⁺ from both galactose-free and galactose-containing reactions. These characteristics of the enzyme of HVJ were similar to those of mammalian particulate enzyme [1]. Guanylate cyclase activity in HVJ was not activated by NaF and trimethylphosphite, compounds that widely activate the guanylate cyclase activity from rat tissues [15].

Fig. 8. Effect of anti-nucleoplasmin serum on guanylate cyclase activity of HVJ (●) and particulate fractions of embryonated egg allantoic membranes (○) and of rat liver (●). After incubation of enzymes and immunoglobulin fractions in the reaction mixture for guanylate cyclase activity except substrate at room temperature for 30 min, the reactions were started by adding Mg²⁺. Effects of anti-serum on soluble guanylate cyclase activity was also examined and no effect was observed (not shown). anti-nucleoplasmin serum (——), control serum (———).