ACTIVATION OF THE SENDAI VIRUS FUSION PROTEIN BY RECEPTOR BINDING

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2,3 Dehydro-2-deoxy-N-acetyl-neuraminic acid (DNANA) competitively inhibits the neuraminidase activity of Hemagglutinin-neuraminidase (HN) from Sendai virus. The inhibition constant depends on the presence of the Fusion (F) protein,which is 30 μM in the presence of active F protein and 50 μM when the F protein is inactivated. These data correlate with previously reported evidence of interaction of the F protein with HN (Dallocchio, F., Tomasi,M., & Bellini, T. (1994) Biochem. Biophys. Res. Comm. **201**, 988-993). Desialyzation of erythrocytes, by Clostridium neuraminidase, lowers the hemolytic activity of SV to <0.1% of that observed on untreated erythrocytes.However, addition of DNANA causes a concentration-dependent increase of hemolytic activity. Both HN and the F protein are required for the activation of hemolytic activity by DNANA. The affinity constant for DNANA, calculated from the activation of hemolytic activity on desialyzed erythrocytes, is 35 μM, very close to the Ki for neuraminidase activity. These data suggest that the binding of the F protein to HN, induced by the binding to HN of a substrate or a substrate analogue, causes a conformational change which activates the F protein. © 1995 Academic Press, Inc.

The envelope of paramyxoviruses contains two glycoproteins, Hemagglutinin-neuraminidase (HN) and the Fusogenic (F) protein. There is considerable evidence to suggest that HN, together with the receptor-binding and receptor-destroying activities, also plays a role in the fusion process. Miura et al. (1) and Portner et. al. (2) reported that monoclonal antibodies, inactivating HN, inhibit syncitia formation and hemolysis, even if the hemagglutinating activity is mantained. Moscona e Peluso (3) have shown that a cell line permanently infected by virus does not form syncitia, because these cells are deprived of sialic acid, but form syncitia when mixed with cells bearing sialic acid. Hu et al. (4) showed that cells expressing the F protein from PIV3 did not form syncitia, unless HN from the same virus was coexpressed on the same cells. The coexpression of F from PIV3 and HN from PIV2, or F from PIV2 and HN from PIV3, did not induce

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syncitia formation. In a similar experiment (5), fusion was activated by coexpression of PIV1 HN and the Sendai virus F, while coexpression of the Sendai virus HN and PIV1 F does not cause cell fusion. Although in some cases (6) virus-cell fusion has been reported in the absence of active HN, at least a facilitating role of HN in the fusion event is generally accepted (7). The current theory is that HN ensures the correct distance between the membranes to allow fusion to take place (8).

We have reported several istances that HN, once bound to the receptor, displays an allosteric inhibitory site (9), and that the physiological effector is the F protein (10-11). This evidence of a receptor-mediated interaction between HN and F suggests that the fusogenic activity of F could be triggered by interaction with HN. Other authors (12-13) have also suggested that HN-mediated conformational changes lead to activation of the fusogenic activity of F. In the present report, we will show that the binding of HN to its receptor directly correlates with the activation of the fusogenic activity of F.

Materials and methods

Sendai virus, Z strain, was grown in the allantoic sac of 10-day-old embryonated chicken eggs, harvested 48 h after injection, and purified as described (14). The virus was resuspended in 10 mM phosphate buffer, pH 7.4, 140 mM NaCl (PBS), and stored at -70 °C. Viral protein concentrations were determined by a modified Lowry procedure (15). 2,3 Dehydro-2-deoxy-N-acetyl-neuraminic acid (DNANA) was purchased from Boehringer. Neuraminidase activity was assayed in PBS using sialyl lactose as substrate, and the release of neuraminic acid was assayed by the thiobarbituric acid procedure (16). To detect the allosteric site, the assay was carried out at 4 °C, using fetuin as substrate (9).

To inactivate F protein, the virus was treated with trypsin (TPCK treated, Sigma), 100:1 by weight, at 37 °C for 30 min. The reaction was stopped at the desired time by adding a 10 fold molar excess of bovine pancreatic trypsin inhibitor (Sigma). The virus was freed from trypsin and the trypsin inhibitor by centrifugation in a Beckman TL 100 centrifuge (10 min, 95000 rpm); the viral pellet was resuspended in PBS and washed twice with PBS by centrifugation. A control experiment was carried out by adding the trypsin inhibitor to the virus before adding trypsin. HN was inactivated by incubation with 5 mM DTT for 30 min at 37 °C. Residual DTT was removed by centrifugation, and the F protein was reactivated by overnight spontaneous air oxidation at 22 °C.

The water-soluble C-terminal fragment of HN (cHN) was prepared as described previously (17)

SDS-PAGE was carried out according to Laemly (18). The gels were either stained with Coomasie Blu R 250, or blotted on nylon membranes (Schleicher & Schuell) and detected with ConA-peroxydase (19).

Sialic acid receptors on erythrocytes were destroyed by incubation with Clostridium perfringens neuraminidase (Boehringer). Erythocytes were washed three times in PBS, and resuspended at 60% hematocrit, then 0.5 ml of cell suspension was incubated overnight with 0.15 IU of neuraminidase. Cells were washed in PBS by centrifugation. Hemagglutination was assayed as previously described (10). Hemagglutination of desialized erythrocytes was not observed with Sendai virus up to 3 mg/ml. Hemolytic activity on desialized erythrocytes was assayed by measuring the release of hemoglobin spectrophotometrically at 405 nm. The assay was carried out at 37 °C, with 0.4 mg/ml of viral proteins.

Results and discussion

Inhibition of the neuraminidase activity by DNANA.

D-NANA inhibits the neuraminidase activity of cHN, the soluble C-terminal fragment of

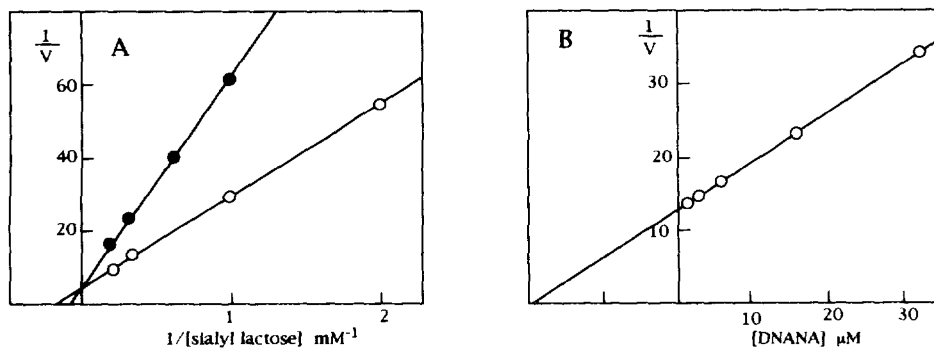

Figure 1. Inhibition of cHN by DNANA. **A**: The neuraminidase activity of cHN was assayed at 37 °C in PBS, pH 7.4, in the absence (○) or in the presence (●) of 50 mM DNANA. **B**: Inhibition of cHN activity by different concentrations of DNANA. Enzymatic activities were measured with 3 mM sialyl-lactose as substrate, and the inhibitor at the concentrations indicated in abscissa.

HN, obtained by selective trypsin digestion (17). The inhibition is competitive with the substrate (Fig. 1A), with K_i = 51.2 μM (Fig. 1B). Virus-bound HN is also competitively inhibited by D-NANA, with K_i =31 μM. The increased affinity of HN for the inhibitor, when compared to cHN, is not dependent on structural differences in the protein: in fact, selective inactivation of the F protein (20) causes the K_i of virus-bound HN to increase to 55 μM, a value close to that observed with cHN (Fig.2). The effect of F on the affinity of HN for the inhibitor cannot be explained by an interaction of D-NANA with F, which does not bind to sialic acid containing receptors. Taking into account that the substrate causes the binding of F to HN (9-11), a similar event can be triggered by D-NANA. Thus while HN in the presence of inactive F, like cHN, allow the determination of the same true inhibition constant, the presence of active F displaces the equilibrium:

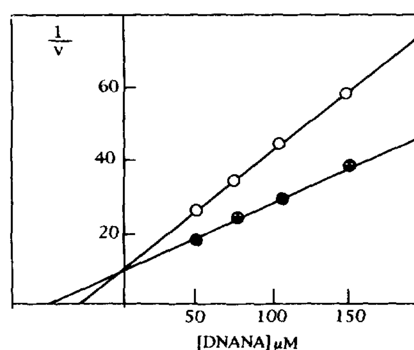

Figure 2. Inhibition of virus-bound HN by DNANA. The assay conditions were the same as described in Figure 1. (○), untreated virus in the presence of inhibitor; (●), trypsin-treated virus in the presence of inhibitor.

The presence of an additional HN-DNANA-F inactive complex shifts the equilibrium toward the inactive species, and leads to the determination of an apparent K_i, lower than the true K_i.

The differences between the K_i measured in the absence of active F, and the K_i observed in the presence of active F, quantitatively agree with the inhibitory effect of F on the neuraminidase activity of the virus. In fact, in the presence of F, the neuraminidase activity is about 30 % inhibited (11), meaning that the ratio [HN-F]/[HN], in the presence of saturating substrate, is about 0.4. The apparent K_i for DNANA is:

$$K_{iapp} = \frac{[DNANA][HN]}{[DNANA\text{-}HN] + [DNANA\text{-}HN\text{-}F]}$$

substituting [DNANA-HN]+[DNANA-HN-F] with [DNANA-HN](1+0.4), then:

$$K_{iapp} = \frac{[DNANA][HN]}{[DNANA\text{-}HN]\ (1+0.4)}$$

thus $K_{iapp} = K_i / 1.4$, and this will cause the measured K_i to decrease from 51 μM, in the absence of active F, to 36 μM in the presence of active F. The quantitative agreement, within the experimental error, between the inhibition of the neuraminidase activity by F and the effect of the same protein on the inhibition constant of DNANA, strongly supports the hypothesis that the differences in the K_i are a consequence of the binding of F to HN induced by the inhibitor.

The differences in the measured K_i, caused by the presence of active F, suggested that the inhibitor DNANA can play all the roles of the true substrate. If these interactions are involved in the activation of the fusogenic activity of F, then DNANA might promote the fusion of the virus with cells lacking sialic acid receptors. Thus we measured the hemolytic activity of Sendai virus with desialized erythrocytes.

The hemolytic activity of SV with desialized erythrocytes is extremely low, and proceeds only during the first few minutes of incubation. This hemolytic activity could be due either to a very low level of receptors not destroyed by the neuraminidase, or to some aspecific interaction. The presence in the incubation mixture of DNANA causes a concentration-dependent increase of hemolytic activity, and the hemolysis proceeds with increasing incubation times (Fig.3A). This suggests that the limiting step in the DNANA-promoted reaction is the correct encounter between virions and erythrocytes. The effect of DNANA is saturable, with half-maximal activation at 37 μM (Fig.3B), a value very close to the K_i of DNANA for HN. The inactivation of F by trypsin digestion, or alternatively the inactivation of HN by reduction with DTT, both completely suppress any effect of DNANA (not shown). This indicates that the activation of fusogenic activity by DNANA requires the presence of both proteins.

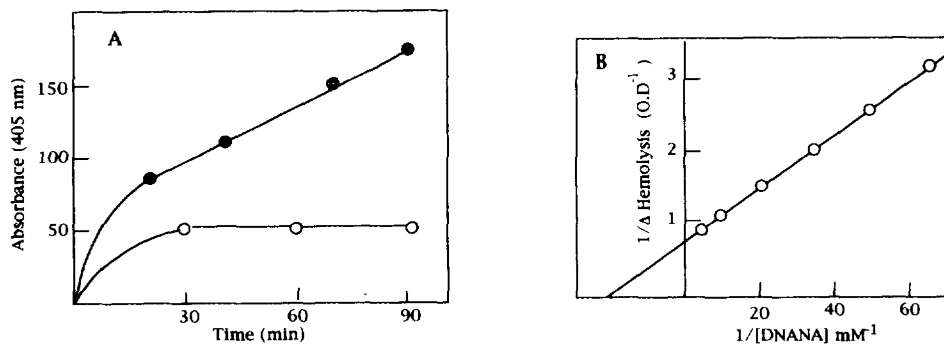

Figure 3. Activation of the hemolytic activity on desialized erythrocytes by DNANA. **A**: The hemolytic activity, measured as described under Methods, was assayed at the times indicated in abscissa in the absence(○)and in the presence (●) of 100 mM DNANA. **B**: the reciprocal of the increase of hemolytic activity, induced by DNANA, is plotted against the reciprocal of DNANA concentrations.

These data suggest that the binding of a receptor, mimicking agent to HN not only causes the formation of an HN-F complex, but also that the formation of this complex induces a conformational change on F which activates fusogenic activity.

If the experimental data here reported are correctly interpreted, then a set of information is already available on the specific requirements for the fusion of SV and other paramyxoviruses with cells, and in particular the need for active HN (1,2), for receptors on the surface of host cells (3), and for the correct pairing of HN and F (4-5), can be easily explained by the following multistep mechanism:

1- HN binds to the sialic acid receptor and undergoes a conformational change, exposing the allosteric site;

2- F binds to the allosteric site of HN, inhibiting neuraminidase activity and strengthening the binding between virus and cell;

3- by interacting with HN, F too undergoes a conformational change, which probably consists of exposing the fusogenic N-terminal peptide.

At this point ,fusion between the viral envelope and cell membrane occur.

In this context, the interaction of F with HN plays the same role as that played by the influenza virus with the pH change, causing a conformational change on the Hemagglutinin and exposing the fusogenic N-terminal peptide. We can hypothesize that many viruses which fuse with a pH-independent mechanism could have a similar cooperative mechanism between receptor binding and fusion activation, and some recent reports on HIV (21), which fuses with a pH independent mechanism, support this hypothesis.

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