

Peptides derived from the heptad repeat region near the C-terminal of Sendai virus F protein bind the hemagglutinin-neuraminidase ectodomain

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Abstract Previously, we showed that Sendai virus fusion protein (F) acts as an inhibitor of neuraminidase activity of hemagglutinin-neuraminidase (HN) protein. Here we report that synthetic peptides derived from the heptad repeat region proximal to the transmembrane domain (HR2) of Sendai virus F inhibit fusion and enhance the enzymatic activity of the HN. This occurs on the virus-bound HN and on its soluble globular head. The enhancing effect on virus-bound HN is reversible and depends on the presence of F. The data indicate that, by binding to the HN ectodomain, the HR2 peptides abolish the F inhibition of HN and disrupt the communication between the F and HN essential to promote virus–cell fusion.

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1. Introduction

Fusion-promoting proteins from several viruses are synthesized as inactive precursors, which are processed by specific proteases forming two subunits, one involved in the fusion process, which is activated at the time of the fusion. The conformational changes needed to activate the fusion proteins (F) are triggered by different events, like pH change or receptor binding. It has been suggested that the receptor-binding subunits act as a clamp to keep the fusion proteins in the inactive conformation, and that the binding of the receptor releases the fusion protein which acquires the most stable, fusion-active conformation [1].

In the paramyxoviruses the mechanism of triggering of the fusion is poorly understood. Nevertheless it has been shown that the fusion requires the presence of hemagglutinin-neuraminidase (HN), a separate protein carrying the receptor-binding activity [2], and we have shown [3] that in Sendai virus, a paramyxovirus, the binding of HN to a soluble receptor promotes the fusion of the virus with receptor-deprived cells. Therefore also in paramyxoviruses receptor binding has

a key role in the fusion process, however, it appears that the trigger for the fusion comes from the interaction between HN and F, rather than from the dissociation of proteins/subunits bearing the receptor-binding and fusion-promoting activities.

The fusion-promoting proteins from several unrelated viruses have two 4–3 heptad repeat (HR) sequences, one near the N-terminal fusion peptide (HR1), and the other near the C-terminal transmembrane domain (HR2) [4]. Recent reports have shown that synthetic peptides, corresponding to the HR2 region, inhibit the fusion process [5–10]. Since in paramyxovirus the activation of the fusion requires the interaction between HN and F, we explored the possibility of an interaction of the synthetic peptides, containing the HR2 sequence, with the receptor-binding protein HN. This kind of experiment can be easily carried out with Sendai virus, where the receptor-binding activity is located on a protein, HN, which can be obtained in soluble form (cHN) by selective proteolytic cleavage [11]. In the present report we show that inhibitory peptides bind both to cHN and to HN, and that the cHN binding and fusion inhibition have the same requirements.

2. Materials and methods

2.1. Sendai virus preparation

Sendai virus was grown in the allantoic cavity of 10 day embryonated eggs, and purified as previously described [12]. The F protein was inactivated by incubation of the virus with trypsin (2% w/w) for 30 min at 37°C. Reconstituted Sendai virus envelopes were prepared from intact and trypsin-inactivated virus as previously described [13]. Liposomes containing HN were prepared from trifluoperazine-solubilized HN and chloroform/methanol-extracted egg lipids, keeping the phospholipid/protein ratio similar to that reported for the virus (276 nmol of phospholipid/mg protein [14]).

2.2. HN C-terminal fragment preparation

The water-soluble, C-terminal fragment of HN, cHN, was prepared with a new procedure. Briefly, the virus was suspended in 10 mM MES buffer, pH 6.0, containing 150 mM NaCl, and HN was selectively solubilized with trifluoperazine. The nucleocapsid with the insoluble F protein was sedimented by centrifugation at 1000×g, and the clear supernatant was treated with trypsin (5% by weight of the viral proteins) for 20 min at room temperature. The solution was passed through a column of Amberlite XA2 (9×1.7 cm) to remove the detergent, and the flow-through was poured into a phosphocellulose column (9×1.7 cm) equilibrated in 10 mM MES, 150 mM NaCl, pH 6.0. The unadsorbed material, containing cHN, was concentrated by centrifugation on Centricon CF25 (Amicon), and appeared as a single band on SDS–PAGE.

The cHN concentration was determined by the absorbance at 280 nm, with an $E_{1\%}^{1\text{cm}}$ of 10.83 [15].

2.3. Peptide synthesis

Peptides were synthesized by solid phase methods, using an automated continuous-flow peptide synthesizer. The stepwise synthesis was

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Abbreviations: F, fusion protein; HN, hemagglutinin-neuraminidase; cHN, soluble ectodomain of HN; HR1, heptad repeat region proximal to fusion peptide of F protein; HR2, heptad repeat region proximal to transmembrane domain of F protein; NA, neuraminidase activity; HE, hemolytic activity

carried out by Fmoc/tBu chemistry. The Na-Fmoc amino acids (4 equiv.) were condensed using DIPCDI (*N,N*-) (4 equiv.) and HOBt (1-hydroxybenzotriazole) (4 equiv.) as coupling agents for 1 h starting to functionalized Wang resin. The peptides were purified by high-performance liquid chromatography.

2.4. Enzymatic and hemolytic activities

Enzymatic activity was assayed at 37°C, using sialyl-lactose as substrate, by measuring the release of sialic acid with the thiobarbituric acid assay [16].

Hemolytic activity (HE) was detected by incubating, in 20 mM Tris–HCl, 140 mM NaCl, pH 7.4 (TBS) at 37°C, viral particles with 2% human red blood cells (type 0, Rh+) obtained from healthy donors who had given informed consent. Samples were then diluted five-fold with cold TBS, and intact cells were removed by centrifugation in a bench centrifuge. The released hemoglobin was evaluated as supernatant absorbance at 413 nm [12]. 100% hemolysis was determined by addition of Triton X-100, 1% final concentration, to an identically treated sample.

2.5. Peptide affinity

The affinity of the peptides for cHN was determined kinetically. The dependence of the enzymatic activity from the peptide concentration is:

$$\frac{v}{v_p - v} = \frac{1}{\alpha - 1} \left(1 + \frac{K_p}{[P]} \right)$$

where v and v_p are the reaction rates measured in the absence and in the presence of the peptide(s), α is the ratio between the catalytic rate constants in the presence and in the absence of saturating peptide, K_p is the (kinetically derived) dissociation constant of the peptide from cHN, and $[P]$ is the concentration of the peptide. When the activation requires the binding to cHN of n peptide molecules, additional powers up to $(P/K_p)^n$ must be added.

3. Results

3.1. Effect of synthetic peptides on Sendai virus hemolytic activity

It has been previously shown that hemolytic activity of Sendai virus is inhibited by the peptide 473–495 from the C-terminal HR region of the F protein [7], and more efficiently by the peptide 465–495, containing one more repeat [17]. In our experiments we used a 20 amino acid peptide within the same sequence (residues 473–492, FLQDSKAELEKARKIL-SEVG). As a control we tested the peptide KKELSLFLGS-RIVEADKAQE, derived from the same HR2 peptide but with a scrambled sequence, and a 28 amino acid peptide derived from the HR2 region of the mumps virus (residues 453–480, TELSKVNASLQNAVKYIRESNHQLQSVS, strain Gloucl/UK96). Since the peptide is slightly shorter than that previously described, we first assayed the effect on the hemolytic activity of Sendai virus. The peptide with the sequence of the Sendai virus F protein causes 50% inhibition of the hemolytic activity at 17 μ M, while both the scrambled peptides and the heterologous peptide from the F protein of the mumps virus did not inhibit the hemolytic activity of the Sendai virus.

3.2. Effect of the peptide on the enzymatic activity of cHN and viral native HN

When the same peptides are tested with the soluble form of the viral neuraminidase, cHN, Sendai virus-derived peptide causes a remarkable activation of the neuraminidase activity (NA) at micromolar concentrations, while the scrambled version of the same peptide (up to 0.2 mM) and the peptide from mumps virus (up to 0.3 mM) do not show any effect.

To verify whether peptide acts by modifying the catalytic

activity or by changing the affinity for the substrate, the enzymatic activity was measured at different substrate concentrations. The double reciprocal plot shows that peptide does not modify the K_m for the substrate, but only the V_{max} (not shown). This observation indicates that the peptide does not affect the affinity of HN for the sialic acid-containing receptors, and agrees with the observation that the peptide does not modify the hemagglutinating activity, as previously reported for other peptides containing the HR sequence of the F protein.

The binding affinity of the peptide for cHN was determined by measuring the extent of activation as a function of the concentration of the peptide. The plot of the reciprocal of the relative increase of the neuraminidase activity against the reciprocal of the peptide concentrations is not linear (Fig. 1). The best fit of the experimental data was obtained with a third-order polynomial, suggesting that in the activated state cHN bear three peptide molecules. At least two models, kinetically indistinguishable, can fit the experimental data. Either the peptide self-associates with a K_{ass} of 1.00×10^{17} M $^{-1}$, and the trimer binds to cHN with a K_d of 4.69×10^{-7} M, or three peptide molecules independently bind cHN, each with a K_d of 1.67×10^{-8} M.

The peptide activates not only soluble cHN, but also the virus-inserted HN. However, the extent of activation of the enzymatic activity of the virus-inserted HN is dependent on the presence of the F protein. In the intact virus the peptides cause about a 100% increase of the enzymatic activity, while in the trypsin-treated virus, where the F protein is inactivated by a selective cleavage at arginine 413 [18], the peptides cause only a 35% increase of the enzymatic activity (Fig. 2A). To verify whether trypsin treatment modifies the response of HN to the peptide, the activation of the enzymatic activity of HN in trypsin-treated virus was compared with the activation of purified HN inserted into liposomes. In both cases (Fig. 2A) the extent of activation induced by the peptide is the same,

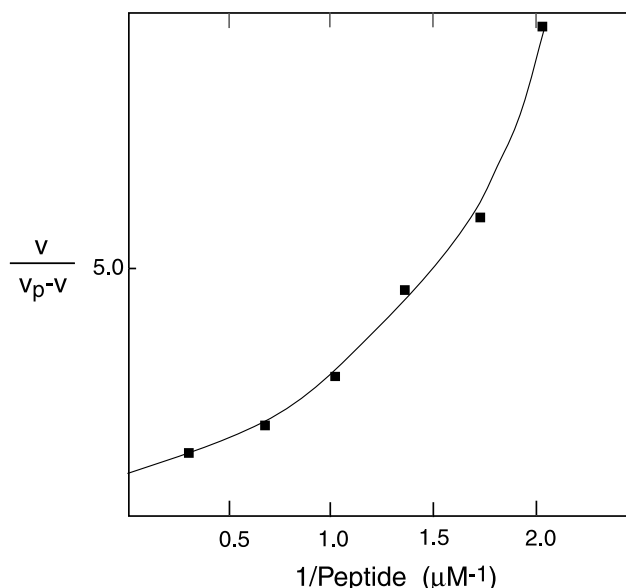


Fig. 1. Effect of the peptide concentrations on cHN activity. The neuraminidase activity of cHN was assayed in the absence and in the presence of the peptide, and the reciprocal of the relative increase of the neuraminidase activity is plotted against the reciprocal of the peptide concentrations.

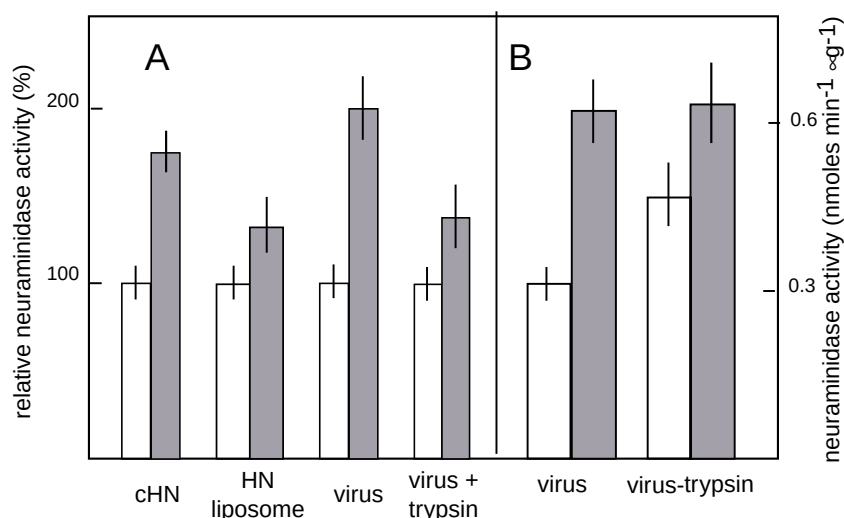


Fig. 2. Effect of the peptides on the neuraminidase activity of membrane-bound HN. A: Relative increase of the neuraminidase activity induced by 10 μ M peptide. The activity of HN in the absence of peptide is normalized to 100% in each condition (liposomes, virus, trypsin-treated virus). White bar: HN; gray bar: HN plus peptide. B: Specific activity of HN in untreated and trypsin-treated virus. White bar in the absence of peptide; gray bar in the presence of peptide.

meaning that the trypsin modifies the response of HN only by inactivating F.

The description of these experiments changes if we look at the absolute values of the enzymatic activity, instead of at the percent activation by the peptide. Since in trypsin-treated virus the neuraminidase activity is higher than the activity in untreated virus, a low activation on a more active enzyme and a high activation on a less active enzyme yield the same enzymatic activity (Fig. 2B). The increase of the neuraminidase activity caused by trypsin destruction of F has already been observed, and it has been shown that F acts as an allosteric inhibitor of HN [19]. The data suggest that the peptide has a dual effect on viral HN: it causes a direct activation of the enzymatic activity, and suppresses the inhibition generated by the F protein. Since in the trypsin-treated virus the allosteric inhibition is already suppressed, the peptide produces only a lower activation.

3.3. Reversibility of peptide effects

The observation that the HR2 peptide suppresses the inhibition of HN by F suggests that the peptide could inactivate F. This hypothesis is very likely, in fact the HR2 peptide is thought to exert its antiviral activity by forming an irreversible abortive complex with the fusion-promoting proteins. However, the interaction of HR2 peptides with the fusion proteins is thought to occur during the irreversible transition from the non-fusogenic to the fusogenic state. Since under our experimental conditions there is no evidence of such a transition, we tested the reversibility of action of the HR2 peptide on both HN activation and hemolytic activity inhibition.

To verify whether the activation of the neuraminidase activity of the viral HN by peptides is reversible, the virus was incubated with the peptide and then washed two times by centrifugation. Although the recovery after washing is only 60–70%, both the activation of the neuraminidase activity and the inhibition of the hemolytic activity appear fully reversed by washing (Table 1). The recovery is better evidenced by the ratio of the activities in peptide-treated and control virus. This means the peptide does not cause the irreversible inactivation of the F protein.

4. Discussion

The data reported here clearly indicate that a fusion-inhibiting peptide, derived from the HR2 region of the F protein, reversibly interacts with HN. Moreover, this peptide suppresses the already described inhibition of the neuraminidase activity by F [19]. We have already shown that an allosteric inhibitory site appears on HN upon binding to the substrate [20], and that F inhibits the neuraminidase activity of HN, suggesting that F is the physiological inhibitor of HN [19]. The inhibition of HN appears to correlate with the activation of F, in fact the binding of a receptor analogue, which discloses the allosteric site, also promotes the fusion of the virus with receptor-deprived erythrocytes [3].

The binding of the HR2-derived peptide to HN and cHN suggests that HN–F interactions can occur through the HR2 region of the fusion protein. The binding of HN to the HR2 region of F is consistent with the structure of the F protein that, although unknown, is reasonably similar to that of F from Newcastle disease virus. In the three-dimensional structure of the F protein from Newcastle disease virus [21] the HR2 is not visible, suggesting that this part of the protein is poorly structured or, at least, not embedded in a rigid structural fold. However, an interaction of the HN ectodomain with the HR2 region of F (very close to the transmembrane region) requires that the globular head of HN lies close to the viral membrane. Although this could appear unlikely, it

Table 1
Reversibility of the activation by peptides

	Before washing			After washing		
	NA	HE	NA/HE	NA	HE	NA/HE
Virus	100	100	1.0	65	61	1.06
Virus+peptide	159	72	2.21	67	70	0.96

should be remembered that early electron microscopy studies were unable to clearly show spike structures on purified lipid-inserted HN [23,24]. In the paper of Hosaka and Shimizu [23], the virusomes, containing only isolated HN, appeared without spikes, while Hsu et al. [24] reported electron microscopy pictures of the reconstituted HN protein showing knob structures on the lipid surface lacking any kind of stalk. Afterwards, as far as we know, no additional data visualizing the HN stalk have been reported, and we propose that the HN ectodomain is connected to the transmembrane domain through a flexible protein region.

The binding of the HR2 peptide to HN is independent of the presence of the substrate (or substrate analogue) and activates the neuraminidase activity, while activation of F appears to require the binding of the substrate and the inhibition of the enzymatic activity. Therefore the binding of HR2-derived peptide evidences a different interaction between HN and F.

The interactions between HN and F, and the effects of the HR2-derived peptide, can be summarized in Fig. 3. The binding of F to HN through HR2 is likely to occur randomly on the envelope, keeping part of the HN and F together (Fig. 3A). When the virus binds a receptor on the cell surface, HN undergoes a conformational change allowing a different interaction with F, resulting in the inhibition of the neuraminidase activity and in the activation of F. At this point the HR2 region of F could be free to form the six-helix bundle observed in the fusion-active conformation (Fig. 3C).

In light of the above suggested mechanism, the binding of HR2 peptides to HN, competing with the direct binding of the HR2 region of F, prevents the correct reciprocal orientation of HN and F (Fig. 3B), and consequently precludes the inhibition of HN by F (Fig. 3D). In fact, in the virus, the main contribution to the increase of the neuraminidase activity induced by HR2 peptide comes from suppression of the inhibition by F.

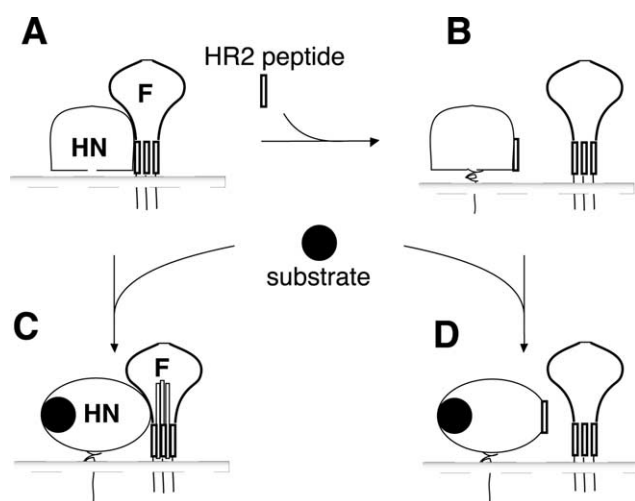


Fig. 3. Early stage of Sendai virus-cell interaction. The HN enzyme ectodomain interacts with the HR region proximal to the transmembrane domain of F protein (HR2) (A); when the HN ectodomain binds to sialic acid, it induces a conformational change of F (C). The synthetic HR2 peptide prevents the HN interaction with F (B) and the conformational change of F as well, in the presence of substrate-enzyme complex (D).

A quite surprising observation is that the mechanism depicted to explain the effect of HR2 peptide on Sendai virus HN is not conflicting with some recently published experiments on Sendai virus 5, a paramyxovirus which can also fuse in the absence of HN.

It has been shown that fusion inhibition by HR1 peptides at low temperatures occurs only when HN is present, and furthermore the extent of fusion in the absence of HN is close to that observed in the presence of HN and HR1 [22]. If we suppose that the HR2 region of F is unstructured and functionally binds HN, the HR1 peptides can associate with the HR2 region and disrupt the HN-F interaction, lowering the fusion near to the levels observed in the absence of HN. In the same report it is observed that HR1 peptides decrease the number of bound red blood cells to the cells expressing the HN and F from Sendai virus 5. This observation could be explained by an increase of the neuraminidase activity of HN. These data agree with the observation that the uncoupling of HN from F, induced by HR2 peptides, increases the neuraminidase activity mainly by suppressing the inhibition by F.

In conclusion, the HR regions of the F protein of paramyxoviruses appear involved in both the early and late events of F activation. While the main inhibitory effect of HR peptides appears related to the perturbation of the correct refolding of F from the inactive to the active conformation, HR2 may also be involved in the early triggering of F activation mediated by the receptor recognition.

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