Biochemical analysis of *Hyphantria cunea* NPV attachment to *Spodoptera frugiperda* 21 cells

Jin O Park¹, Kyung Hwa Chang¹, Hyung Hoan Lee³ & In Sik Chung^{1,2,*}

¹ Department and ² Institute of Genetic Engineering, Kyung Hee University, Suwon, 449-701, Korea

(* Author for all correspondence, e-mail: ischung @ nms.kyunghee.ac.kr)

³ Department of Biology, Kon Kuk University, Seoul, 133-701, Korea

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Abstract

Binding characteristics of *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) to *Spodoptera frugiperda* 21 (Sf21) cells was determined. The cells displayed an affinity of $0.9 \times 10^{10} \text{ M}^{-1}$ with about 8900 binding sites per cell. The biochemical nature of HcNPV-binding sites on the cell surface was also partially elucidated. There were 45 to 49% reductions in HcNPV binding following the pretreatment of cells with three proteases, suggesting the involvement of a cellular protein component in virus binding. Tunicamycin, which inhibits N-linked glycosylation and the expression of some membrane proteins on the cell surface, reduced virus binding suggesting a role for glycoprotein(s) in binding. Treatment of cells with wheat germ agglutinin or neuraminidase did not measurably reduce virus binding, indicating that oligosaccharides containing N-acetylglucosamine or sialic acid are not directly involved in HcNPV attachment. The negative effect of methylamine on HcNPV binding seems to be due to the fact that HcNPV entry via an endocytic pathway is blocked by the increased pH of the endosome. Data on energy inhibitors (sodium azide and dinitrophenol) indicates that HcNPV attachment to Sf21 cells may be closely linked to viral entry via receptor-mediated endocytosis. These findings suggest that the binding site moiety has a glycoprotein component, but that direct involvement of oligosaccharides containing N-acetylglucosamine or sialic acid residues in binding is unlikely, and that HcNPV attachment to Sf21 cells might be via receptor-mediated endocytosis.

Abbreviations: HcNPV – *Hyphantria cunea* Nuclear Polyhedrosis Virus; LdNPV – *Lymantria dispar* Nuclear Polyhedrosis Virus; NOV – Non-Occluded Virus; OV – Occluded Virus; SD – standard deviation; Sf21 – *Spodoptera frugiperda* 21; TM – Tunicamycin; WGA – Wheat germ agglutinin

Introduction

Insect viruses have received considerable attention because they have potential as safe and effective biological control agents. The nuclear polyhedrosis viruses (NPVs) are an important subgroup of pathogenic baculoviruses that may prove to be valuable insect control agents. Fall webworm (*Hyphantria cunea*), an occasional pest on fruit and shade trees, is a serious pest in Korea. *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) was isolated from dead larvae near Seoul and purified (Choe et al., 1986). The HcNPVs were used to infect *Spodoptera frugiperda* 21 (Sf21)

in vitro (Lee, 1987; Lee and Lee, 1988). It is a rod-shaped virus with a double-stranded and circular DNA genome (Lee and Lee, 1991). There are two forms of the HcNPV virus: occluded virus (OV) and non-occluded virus (NOV). In tissue culture, NOV is more infectious than OV. The majority of the NOVs in NPV have been observed to enter cells *in vivo* by fusion at the cell surface (Wang and Kelly, 1985). However, the infectious entry route into tissue culture cells has been shown to be primarily through receptor-mediated endocytosis (Wickham, 1992); a minority appeared to enter through some other route, possibly by fusion at the cell surface (Volkman et al., 1986).

Attachment of virus particles to specific receptors on the plasma membrane is one of the initial events in the interaction of NPV with insect cells. The attachment kinetics of baculoviruses *Lymantria dispar* NPV (Horton and Burand, 1993) and *Trichoplusia ni* NPV (Wang and Kelly, 1985) have been investigated. However, to our knowledge, the attachment process of HcNPV has not yet been studied. As important characteristics in attachment processes, the number or affinity of HcNPV binding sites may be quite different from those of LdNPV and TnNPV. In addition, it is unclear whether the biochemical nature of HcNPV binding sites is identical among insect cell species. To answer these questions, we investigated the binding characteristics of HcNPV to Sf21 cells and the biochemical nature of binding sites on the cell surface.

Materials and methods

Virus, cells and media

The virus studied was *Hyphantria cunea* nuclear polyhedrosis virus, which was described by Lee (1987). The virus inoculum used was NOV derived from cell culture media (Lee and Miller, 1978). The *Spodoptera frugiperda* cell line (IPLB-SF-21) (Sf21) was obtained from L.K. Miller (University of Georgia, Athens, GA, U.S.A.). Sf21 cells were routinely maintained in TNM-FH (Sigma, St. Louis, MO) medium supplemented with 0.35 g l⁻¹ sodium bicarbonate, 50 µg ml⁻¹ gentamycin (Sigma, St. Louis, MO), 2.5 µg ml⁻¹ fungizone and 10% fetal bovine serum (Gibco, Grand Island, NY). For ³²P-labelled HcNPV, Sf21 cells were infected with HcNPV and the inoculum was replaced by TNM-FH medium minus phosphate containing 10% serum and 10 µCi ml⁻¹ of ³²P-orthophosphate (Amersham, Buckinghamshire, England). The cells were then incubated at 27 °C and the virus was harvested two days before cell lysis occurred. The solution was centrifuged at 1000 x g for 20 min to remove cells and cell debris. Then, the supernatant was concentrated and purified by centrifugation at 8000 x g through a 35% w/w sucrose cushion. The virus pellet was resuspended overnight in TNM-FH medium.

Scatchard analysis

HcNPV binding was measured by adding progressively higher concentrations of labelled virus to cells. ³²P-labelled HcNPV were incubated with a constant

number (4×10^5 cells well⁻¹) of Sf21 cells at 4 °C for 1 h in 24-well plates. The Sf21 cells were heated at 90 °C for 10 min and these cells were used as a non-permissive control for non-specific binding. All the experiments were performed in triplicate for Scatchard analysis. The radioactivity was measured by a scintillation counter (Wallac, 1209 RackBeta, Turku, Finland). The number of bound/free particles against bound particles were plotted for a Scatchard analysis. Virus particle concentration was determined by measuring DNA concentration. Phage λ DNA at a known concentration was used as a standard. The virus particle concentration was calculated from the molecular weight of the viral genome and the measured HcNPV DNA concentration using spectrophotometer (Varian DMS-300, U.S.A.). The concentration of infectious virus was determined by TCID₅₀ (Reed and Muench, 1938) and TCID₅₀ was converted into infectious particle number (David et al., 1992). The virus concentration in virus particles per liter was divided by Avogadro's number to obtain the molar concentration (Yu et al., 1995).

HcNPV-binding studies

All the experiments were performed in triplicate for HcNPV-binding studies. Sf21 cells were treated with enzymes and lectin as follows. We determined the effective concentrations of enzymes and lectin from published data (Wickham, 1991; Jin et al., 1994). The cells were treated with 0.5 mg ml⁻¹ trypsin (Sigma, St. Louis, MO), 1 mg ml⁻¹ α-chymotrypsin (Sigma, St. Louis, MO), 1 mg ml⁻¹ proteinase K (Sigma, St. Louis, MO), or 0.03 U neuraminidase (Sigma, St. Louis, MO) in PBS buffer (pH 6.2) for 1 h at 27 °C and then washed with PBS to remove residual activity. The cells were also treated with 2 mg ml⁻¹ WGA (Sigma, St. Louis, MO) without and with either trypsin and proteinase K in PBS buffer for 1 h at 27 °C. In the experiments using a glycosylation inhibitor, the cells were preincubated for 48 h with 1 or 4 µg ml⁻¹ tunicamycin (Sigma, St. Louis, MO). To analyze weak base effects, Sf21 cells were preincubated with 15 mM methylamine (Sigma, St. Louis, MO) for 48 h at 27 °C. To study the effect of energy inhibitors, the cells were preincubated with 10 mM sodium azide (Sigma, St. Louis, MO) or 1 mM dinitrophenol (Sigma, St. Louis, MO) for 48 h at 27 °C. The effective concentrations of tunicamycin, methylamine, sodium azide and dinitrophenol were also determined from published data for other AcNPV binding studies

Figure 1. Time-course of specific HcNPV binding to *Spodoptera frugiperda* 21 cells. Heat-treated Sf21 cells (at 90 °C for 10 min) were used as a non-permissive control. Specific binding (●) was obtained by subtracting the non-specific binding (▲) from the total binding (■). Results are presented as the average of triplicates.

(Wickham, 1991; Wickham et al., 1992). The same amount of radio-labelled HcNPV was added to the control and treated cell layers. The cell layer was designed to contain about 4×10^5 cells well⁻¹ in 24-well plates or 1×10^5 cells well⁻¹ in 96-well plates. After 1 h of incubation at 4 °C, the cells and supernatant were separated. The radioactivity was measured in a scintillation counter.

Results and discussion

The time-course of HcNPV adsorption to *Spodoptera frugiperda* 21 (Sf21) cells was studied using a virus binding assay at 4 °C. Heat-treated Sf21 cells (at 90 °C for 10 min) were used as a non-permissive control for non-specific binding. The specific binding value was calculated by subtracting the non-specific binding from the total binding. As shown in Figure 1, specific binding of HcNPV to Sf21 cells occurs notably between 0 and 60 min, but the amount of specific HcNPV binding reaches plateau after about 3 h.

Specific binding data for Sf21 cells were obtained with ³²P-labelled HcNPV. The data transformed into the Scatchard plot showed a straight line, suggesting that a single class of HcNPV-binding sites is present on Sf21 cells. The abscissa intercept of the plot shows that there are about 8900 binding sites per cell (Figure 2). The receptor number on the Sf21 cell surface for HcNPV binding determined in this study was found to be higher than the reported value of 6,000 binding sites per *Trichoplusia ni* 5B1-4 cell for AcNPV (Wickham et al., 1992) and to be lower than that of 10^6 binding sites per *Lymantria dispar* cell for LdNPV (Horton and Burand, 1993). The Sf21 cells had an affinity of 0.9×10^{10} M⁻¹. This affinity value was lower than the reported value (1.6×10^{10} M⁻¹) for the Tn cell line and AcNPV interaction (Wickham et al., 1992).

The biochemical nature of the HcNPV-binding sites on the cell surface was examined by incubating Sf21 cells with three proteases. As shown in Table 1, treatment with trypsin, α -chymotrypsin and proteinase K resulted in 44, 45 and 49% reductions of HcNPV-

Figure 2. Scatchard plot of specific HcNPV binding to *Spodoptera frugiperda* 21 cells. Binding experiments were performed in triplicate at 4 °C for 1 h in 24-well plates. HcNPV binding was measured by adding progressively higher concentrations of 32 P-labelled HcNPV to Sf21 cells (4×10^5 cells well $^{-1}$). Results are presented as the means $\pm$ SD of triplicate runs. Data were analyzed using a linear regression. The coefficient of determination for the fit, r^2 , was 0.94 (significant at 5% level).

Table 1. Effect of enzymes and lectin on HcNPV binding to *Spodoptera frugiperda* 21 cells

Treatment	Concentration	% Virus binding ^a
None		100
Trypsin	0.5 mg ml $^{-1}$	51 ($\pm$ 1)
α -chymotrypsin	1 mg ml $^{-1}$	56 ($\pm$ 0.3)
Proteinase K	1 mg ml $^{-1}$	55 ($\pm$ 3)
Neuraminidase ^b	0.03 unit	99 ($\pm$ 1)
WGA ^c	2 mg ml $^{-1}$	98 ($\pm$ 2)
WGA + Trypsin	2 + 0.5 mg ml $^{-1}$	54 ($\pm$ 3)
WGA + Proteinase K	2 + 1 mg ml $^{-1}$	53 ($\pm$ 9)

^a The percentage virus binding is the average of triplicates.

^b *V. cholerae* neuraminidase.

^c WGA, wheat germ agglutinin.

binding to Sf21 cells, respectively. This indicates that an essential component of the HcNPV-binding moiety on the Sf21 cells is a protein. HcNPV binding to the cells was also investigated using tunicamycin (TM). TM prevents the addition of high-mannose oligosaccharide to N-linked glycoproteins and can also

Table 2. Effect of metabolic inhibitors on HcNPV binding to *Spodoptera frugiperda* 21 cells

Treatment	Concentration	% Virus binding ^a
None		100
Tunicamycin	1 μ g ml $^{-1}$	45 ($\pm$ 1)
	4 μ g ml $^{-1}$	38 ($\pm$ 4)
Methylamine	15 mM	68 ($\pm$ 15)
Sodium azide	10 mM	50 ($\pm$ 9)
2,4-Dinitrophenol	1 mM	52 ($\pm$ 8)

^a The percentage virus binding is the average of triplicates.

inhibit the subsequent expression of glycoproteins on the cell surface (Elbein, 1987). There was a 55–62% reduction in HcNPV binding after treatment of cells with TM (Table 2). The fact that treatment of cells with glycosylation inhibitors such as TM reduced HcNPV binding supports the hypothesis that the cell surface receptor for HcNPV may be a glycoprotein structure. On the other hand, treatment of Sf21 cells with wheat germ agglutinin (WGA) or neuraminidase

had no effect on HcNPV binding. The pretreatment of Sf21 cells with wheat germ agglutinin, which specifically binds to oligosaccharides containing N-acetylglucosamine, did not affect HcNPV binding. WGA experiments combined with either trypsin or proteinase K treatment shows a similar result. In addition, HcNPV binding is insensitive to Sf21 cells treated with neuraminidase, which has the function of desialylation activity. The collective results of the WGA and neuraminidase treatment suggest that an oligosaccharide containing N-acetylglucosamine or sialic acid is not a necessary moiety for the attachment of HcNPV.

The effects of metabolic inhibitors on HcNPV binding are also summarized in Table 2. Inhibition effects were observed with a lysosomotropic agent such as methylamine. The negative effect of methylamine on HcNPV binding is due to the fact that HcNPV entry via an endocytic pathway is blocked by the increased pH of the endosome with weak bases such as methylamine. Endocytosis is an energy-requiring process and thus energy inhibitors such as sodium azide and dinitrophenol have been used as endocytosis inhibitors in the analysis of virus-cell interaction (Kaljot et al., 1988). In our experiments, sodium azide and dinitrophenol strongly inhibited HcNPV binding. Combined with the observed effect of methylamine, the effect of energy inhibitors suggests that HcNPV attachment to Sf21 cells may be closely linked to viral entry via receptor-mediated endocytosis. This agrees with a previous report on the mechanism of *Bombyx mori* NPV entry via receptor-mediated endocytosis in *Bombyx mori* BmN-4 cells (Kim et al., 1997).

This study has provided an insight into the nature of Sf21 cell surface receptors in the HcNPV-insect cell interaction and our findings provide fundamental data for research on bioinsecticide development. However, the entire mechanisms of HcNPV host specificity are still unclear. Further research is needed to resolve the controversy surrounding the nature of insect cell receptors in baculovirus-insect cell interactions and to test whether the host range of baculoviruses is restricted to the level of initial attachment to insect cell receptors.

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