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Binding and fusion of *Autographa californica* nucleopolyhedrovirus to cultured insect cells

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Binding of baculoviruses to insect cells and fusion of the virus envelope to cell membranes are early events suggested to be affected by baculovirus enhancins. The binding of *Autographa californica* nucleopolyhedrovirus (AcMNPV) to the *Spodoptera frugiperda* cell line Sf21 and the fusion of the virus envelope to cell membranes were characterized. Virus binding assays demonstrated that AcMNPV budded virus (BV) bound to specific binding sites on Sf21 cells with an avidity of $2{\cdot}3 \times 10^{10}$ M $^{-1}$. The cells displayed $3{\cdot}1 \times 10^{3}$ specific binding sites per cell in a confluent monolayer. In addition, the effects of pH, buffer composition and cation concentration on the binding were examined. Using a fluorescent probe (R18) and fluorescence microscopy, the fusion of AcMNPV BV envelope to the cell membrane was directly visualized in living cells. It has been reported that *Trichoplusia ni* nucleopolyhedrovirus enters Sf21 cells by membrane fusion at the cell surface; however, the present studies confirmed the well established concept that adsorptive endocytosis is the major entry pathway for baculovirus BV infection. Membrane fusion kinetics and fluorescence microscopy demonstrated and verified that the envelope–cell membrane fusion was triggered by acidification. The effect of a *T. ni* granulovirus enhancin on virus binding and membrane fusion was examined, and no increase in activity was observed.

Introduction

The interaction of an animal virus at the cell surface is critical for establishing infections. Studies on baculovirus binding in cells were reviewed and the fundamentals behind virus–cell binding were clearly elucidated by Hammer *et al.* (1995). There are few comprehensive studies on the binding of baculoviruses to insect cell surfaces. Wickham *et al.* (1992) reported the binding of budded virus (BV) from *Autographa californica* nucleopolyhedrovirus (AcMNPV) to a cell line from *Trichoplusia ni* and showed specific binding of BVs to the cells. Although binding of AcMNPV BV to the *T. ni* cell line was partially characterized (Wickham *et al.*, 1992), the effects of culture conditions such as pH and cation effects on the binding have not been studied. Similar binding studies were conducted for *T. ni* nucleopolyhedrovirus (TnNPV) by Wang & Kelly (1985), but they failed to separate the binding and subsequent internalization, two important and discrete events in the entry of baculovirus into host cells. Consequently, detailed studies on baculovirus BV binding to insect cells are limited to the single report by Wickham *et al.* (1992), who were careful to separate experimentally the temporal events of binding and entry.

The dogma that baculoviruses enter cultured cells by an endocytic pathway has been established by several elegant studies (Volkman, 1986; Leikina *et al.*, 1992; Blissard & Wenz, 1992; Monsma & Blissard, 1995). Although membrane fusion at the cell surface was reported as an alternative method of entry (Volkman *et al.*, 1986), virus entry by this route was at a level of 10000-fold less than that via endocytosis (Volkman & Goldsmith, 1985). However, Kozuma & Hukuhara (1994) have challenged this dogma and, contradicting Volkman (1986) and Blissard & Wenz (1992), they reported that TnNPV entered cultured cells by membrane fusion at the plasma membrane. Clearly, these divergent experimental results need to be resolved.

Studies on fusion kinetics of baculovirus envelope and cell membranes have been reported (Horton & Burand, 1993; Kozuma & Hukuhara, 1994). Unfortunately, in these studies the authors failed to separate experimentally baculovirus-binding kinetics from the kinetics of membrane fusion. Furthermore, it was reported that BV binding (Tanada, 1985; Uchima *et al.*, 1989) and membrane fusion (Kozuma &

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Hukuhara, 1994) are events that can be mediated by baculovirus enhancins (see Corsaro *et al.*, 1993 for review). The fusion studies by Kozuma & Hukuhara (1994) are intriguing in that TnNPV was reported to enter cells by membrane fusion, and this event can be enhanced by the presence of a granulovirus enhancin (called synergistic factor by Kozuma & Hukuhara, 1994). These unusual results suggest that these baculovirus-encoded proteins (enhancins) may have a role in membrane fusion. Recently, enhancins were identified as metalloproteases; however, their mode of action at the midgut level is still being defined (Lepore *et al.*, 1996).

To resolve the contradictions and intriguing studies cited above, we report the binding and fusion of AcMNPV BV to Sf21 cells and the effect of a baculovirus enhancin from *T. ni* granulovirus (TnGV) (Hashimoto *et al.*, 1991) on binding and membrane fusion. The binding studies reported here are consistent with a previous report (Wickham *et al.*, 1992) which demonstrated the presence of specific binding sites on the cell surface. We also report here that the binding was affected by medium composition, pH and bivalent cations. Our findings on the fusion kinetics of baculovirus envelope to cell membranes, which were experimentally separated from the binding event for the first time, confirm earlier findings (Volkman, 1986; Leikina *et al.*, 1992; Blissard & Wenz, 1992) that the fusion of AcMNPV BV envelope to the cell membrane was triggered by acidification. Using a virus labelled with a fluorescent membrane probe (R18), fusion of AcMNPV BV envelopes to cells was observed in living cells by microscopic examination. The direct visualization and kinetic studies of baculovirus–cell membrane fusion confirm the hypothesis that endocytosis is the primary pathway for AcMNPV BV entry (Volkman, 1986), and do not support the recent findings of Kozuma & Hukuhara (1994) that the virus enters cells by membrane fusion at the plasma membrane. Finally, our results reveal that TnGV enhancin has no measurable effect on either AcMNPV BV binding or membrane fusion in cultured cells.

Methods

■ **Viruses and cells.** AcMNPV strain 1A (Wood, 1980) was produced in a *Spodoptera frugiperda* cell line, Sf21 (Vaughn *et al.*, 1977) and grown in either TNM-FH medium with 10% foetal bovine serum (FBS) (Hink, 1970), or in ExCell 400 serum-free medium (JRH BioSciences).

AcMNPV BV used for binding studies was generated by inoculating Sf21 cells in ExCell 400 medium with second passage AcMNPV infectious medium. AcMNPV BV-containing medium was collected after a 2 day incubation of the infected cells at 28 °C. Budded viruses were isolated and purified as described by Wickham *et al.* (1992).

Radioactive AcMNPV BV was prepared as follows: exponential phase Sf21 cells grown in ExCell 400 medium in a 75 cm^2 tissue culture flask were inoculated with 2 ml AcMNPV infectious medium and incubated at 28 °C for 8 h. The cell monolayer was washed twice with methionine-deficient ExCell 401 medium (JRH BioSciences) and incubated in 10 ml fresh methionine-deficient ExCell 401 medium. After 1 h incubation of cells in methionine-deficient medium, the cells were switched to methionine-deficient ExCell 401 medium plus 1 mCi [^{35}S]methionine and incubated at 28 °C. ^{35}S-labelled virus containing medium was collected 40 h post-inoculation (p.i.). The radioactive labelled AcMNPV BV was purified as described above.

AcMNPV BV used for membrane fusion studies was produced in Sf21 cells grown in TNM-FH medium with 10% FBS. AcMNPV BV was purified by sucrose gradient centrifugation (Wood & Speyer, 1978) and labelled with octadecyl Rhodamine B chloride (R18) (Hoestra *et al.*, 1984). Briefly, purified AcMNPV BVs were suspended in 1 ml 10 mM HEPES pH 7·4, 0·85% NaCl at 1 mg protein per ml and mixed with 15 μg R18 (Molecular Probes) in 15 μl ethanol. The virus suspension was then incubated at room temperature in the dark for 1 h. R18-labelled virus was separated from free R18 molecules by centrifugation.

All AcMNPV BV preparations were quantified by measuring viral DNA. Virus concentrations based on physical particles were converted from DNA measurements based on viral genome equivalents (Wickham *et al.*, 1992).

TnGV and TnGV enhancin were produced and purified as described by Wang *et al.* (1994). Enhancin solution was equilibrated into Grace's medium (Grace, 1962) by dialysis against Grace's medium, as described by Hukuhara & Zhu (1989).

■ **Binding assays.** Virus binding assays were conducted by using cell monolayers in 24-well cell culture plates according to Wickham *et al.* (1992). Exponential phase Sf21 cells in ExCell 400 medium were seeded into a 24-well cell culture plate (Falcon) at a density of 6×10^5 cells per well and allowed to attach to the plates for 1 h. The plates were spun at 1000 r.p.m. for 10 min to strengthen the cell attachment, and kept at 4 °C. The medium in each well was aspirated and carefully replaced with 200 μl pre-cooled virus suspension containing a graded concentration series of non-labelled virions in Grace's medium with 1 mg/ml BSA. ^{35}S-labelled virus at 150 particles per cell was included in the non-labelled virus suspensions as indicators for binding quantification. Non-labelled virions equivalent to 1×10^5 virus particles per cell, mixed with ^{35}S-labelled virus at 150 particles per cell, were added to wells for non-specific binding measurement. Binding assays for each virus concentration were carried out in triplicate. After incubation at 4 °C overnight to reach equilibrium binding, the medium was removed and the cell monolayers in each well were carefully washed with 200 μl cold Grace's medium. The cells were then solubilized in 200 μl 1% SDS and collected. Each well was washed once with 200 μl 1% SDS and combined with the first 200 μl of solubilized cells. The radioactivity of the solutions from each well was measured with a Beckman LS liquid scintillation counter. The percentage of virions bound to cells was calculated based on the radioactivities associated with cells versus the total radioactivity added to each well. Specific binding was calculated by subtracting non-specific binding from total binding.

The pH effect on binding of AcMNPV BV to cells was determined as follows: Sf21 cells in ExCell 400 medium were seeded into 24-well plates and incubated at 28 °C until confluent monolayers formed. Cell density was determined by counting cell numbers under a microscope within a calibrated surface area. Cell monolayers in the plates were cooled to 4 °C and washed once with a binding buffer to be used for a specific assay. The binding buffers used were: ExCell 400 medium (from pH 5·7 to 6·8), Rinaldini's solution (Rinaldini, 1954) (from pH 5·7 to 7·0), and 0·2 M sodium phosphate buffer (from pH 5·7 to 7·0). BSA (10 mg/ml) was added to the binding buffers to block non-specific binding. Aliquots of cold binding medium (200 μl) containing 9×10^7 ^{35}S-labelled virions (~ 150 virions per cell) were added to cell monolayers and incubated with virus at 4 °C overnight. Radioactivities associated with cells were determined as described above for competitive binding assays.

The effects of metal cations on virus binding were determined in 20 mM BisTris–HCl buffer with 10 mg/ml BSA pH 6·2, containing a series of concentrations and combinations of NaCl, $CaCl_2$ and $MgCl_2$. The osmolarity of the buffers was adjusted with sucrose to 370–380 mosM using a freeze-point osmometer (Advanced Micro-Osmometer, model 3MO).

Binding of virus to cells in the presence of TnGV enhancin was measured by conducting binding assays in Grace's medium with 10 mg/ml BSA. Cell monolayers in a 24-well plate were incubated at 28 °C for 2 h with TnGV enhancin at two concentrations (25 and 50 μg/ml). Virus binding assays were conducted by using the same method described above, except that TnGV enhancin at 25 and 50 μg/ml was added to the binding medium.

■ **Enzyme treatments.** For protease treatments, Sf21 cell monolayers in a 24-well plate were washed once with PBS and incubated with one of three protease solutions: trypsin (Sigma) in PBS; pronase E (Sigma) in PBS; or dispase (Boehringer Mannheim) in PBS, supplemented with 5 mM $CaCl_2$, and incubated at 37 °C for 20 min. Cell monolayers were carefully washed three times with PBS containing 5 mM PMSF followed by incubation with ^{35}S-labelled AcMNPV BV at 4 °C for 4 h. Virus binding was determined as described previously.

For glycosidase treatments, cell monolayers were treated similarly to the protease treatments, except that glycosidase solutions were added to cells and incubation was performed at 28 °C for 1 h. *N*-glycosidase F (Boehringer Mannheim) was suspended in 0·2 M sodium phosphate buffer pH 7·0. *O*-glycosidase and neuraminidase (both from Boehringer Mannheim) were suspended in 0·2 M sodium phosphate buffer pH 6·2.

■ **Fluorescence microscopy.** Exponential phase Sf21 cells in TNM-FH medium in a 24-well plate were cooled at 4 °C for 30 min. Pre-cooled R18-labelled AcMNPV BV suspension in Grace's medium was added to the cells and incubated at 4 °C for 2 h. After incubation, the cells were quickly washed with cold Grace's medium and replaced with Grace's medium at pH 6·2 or 5·5. The cells were incubated at 28 °C, except when being examined at room temperature using an Olympus inverted fluorescence microscope. To block endocytosis, the cells were pretreated with 0·1% sodium azide in Grace's medium at 4 °C for 1 h before adding R18-labelled virus.

■ **Membrane fusion assays.** Fusion of virus envelopes to cell membranes was measured based on the dequenching of fluorescent dye R18 anchored in the virus envelope (Hoestra *et al.*, 1984). Exponential phase Sf21 cells in TNM-FH medium with 10% FBS were washed with Grace's medium and pelleted by centrifugation at 150 ***g*** for 10 min. The pelleted cells were cooled on ice and then suspended in R18-labelled virus suspension in a small volume at m.o.i. 350–400 virus particles per cell. The cells were kept on ice for 2 h and gently mixed every 15 min. After incubation, the cells were washed with cold Grace's medium to remove unbound virus particles, and centrifuged. The cells with bound virus were suspended in a small volume of Grace's medium on ice. Aliquots of the cell suspension (100 μl) containing $1{\cdot}3 \times 10^6$ cells were added to 3 ml cuvettes containing 1·9 ml Grace's medium at the desired pH values. The cuvettes used were pretreated with a 10% BSA solution. Fluorescence was measured immediately at regular time-points with a Perkin-Elmer fluorescence spectrophotometer (model MPF-44B) with an excitation wave length of 560 nm and emission wave length of 590 nm. Between measurements, the cell suspensions in the cuvettes were kept at 26 °C in the dark. The percentage of membrane fusion was calculated according to Hoestra *et al.* (1984).

■ **Chloroquine and sodium azide treatments.** Sf21 cells were bound with R18-labelled AcMNPV BV. Unbound virus was removed as described above. Aliquots of cell suspension (100 μl) were mixed with 1 μl 10% sodium azide or 100 mM chloroquine, and kept on ice for 30 min. The cells were then suspended in 2 ml Grace's medium containing 0·1% sodium azide or 1 mM chloroquine. Fluorescence dequench assays were performed as described above.

■ **Enhancin fusion assays.** Sf21 cells were bound with R18-labelled virus. Unbound virus was removed as described above. Aliquots of cells were mixed with 50 μg/ml TnGV enhancin, and kept on ice for 30 min. Fusion assays were conducted as described above.

Results

Binding of AcMNPV BV to Sf21 cells and Scatchard analysis

Binding was determined when binding of virus to cells had reached an equilibrium state. The amount of virus particles bound to cells was calculated based on the percentage of radioactivity associated with cells versus the total virus input. Non-specific binding was deducted from total binding to obtain specific binding. The specific binding data from triplicate samples showed a receptor–ligand binding feature (Fig. 1*a*). The physical characteristics of the binding were analysed using Scatchard analysis. The Scatchard plot (Fig. 1*b*) shows that AcMNPV BV binds specifically to Sf21 cells with a dissociation constant coefficient of $4{\cdot}3 \times 10^{-11}$ M, or an affinity of $2{\cdot}3 \times 10^{10}$ M^{-1}. Confluent monolayers of Sf21 cells exhibited $3{\cdot}1 \times 10^3$ specific binding sites per cell.

Binding characteristics

The extent of binding of AcMNPV BV to Sf21 cells at different pH values in sodium phosphate buffer, Rinaldini's solution and ExCell 400 medium was determined. The results showed that the composition of the binding media affected binding. In addition, the effect of pH on binding was altered by the medium composition (Fig. 2). The amounts of virus bound in sodium phosphate buffer and in Rinaldini's solution differed, but binding decreased in both buffers with increasing pH from 5·7 to 7·5. However, the opposite was true in ExCell 400 medium: binding increased with increasing pH from 5·6 to 6·8.

In addition to the existence of specific binding sites on the cell surface, electric charge and divalent cations could also be involved in animal virus binding (Lentz, 1990). To investigate the charge effect, binding of virus to cells was determined in 20 mM BisTris–HCl buffer pH 6·2, using a series of concentrations of metal cations in a constant composition ratio (Na^+:Ca^{2+}:Mg^{2+} at 1:2:2). The results (Fig. 3*a*) showed that virus binding increased and reached a peak when the concentration of cations was increased from 0 to 10 mM. Binding decreased as the cation concentration was increased from 10 to 50 mM. When the cations were increased from 50 to 150 mM, the binding increased and reached a level of binding equivalent to that seen where no metal cations were added. Binding in cation concentrations from 100 to 150 mM was equivalent to the binding observed in Grace's and ExCell 400 media (data not shown).

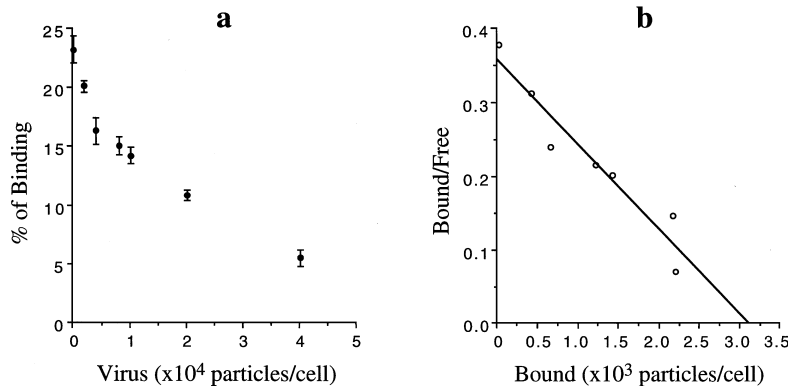

Fig. 1. (*a*) Equilibrium binding assay of AcMNPV BV to Sf21 cells. The binding of AcMNPV BV to Sf21 cells was calculated based on the percentage of binding of ^{35}S-labelled virus to the cells. Error bars represent standard errors from triplicate samples. (*b*) Scatchard plot for binding of AcMNPV BV to Sf21 cells. The specific binding of AcMNPV BV to Sf21 cells was calculated by subtracting non-specific binding of virus to cells from total binding. Data from (*a*) with triplicate samples were averaged and analysed by Scatchard analysis. The slope of the regression line represents $1/K_d$ (K_d = dissociation constant coefficient, $4{\cdot}3 \times 10^{-11}$ M) and the intersection on the *x*-axis represents total specific binding sites on a cell ($3{\cdot}1 \times 10^3$).

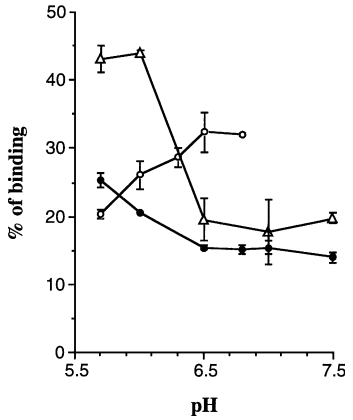

Fig. 2. pH effects on the binding of AcMNPV BV to Sf21 cells. The binding of ^{35}S-labelled AcMNPV BV to Sf21 cells was determined at different pH values in 0·2 M sodium phosphate buffer (△), Rinaldini's solution (●) and ExCell 400 medium (○). Error bars represent standard errors from triplicate samples.

To further study the effect of electric charge and individual cations on virus binding, the binding in buffers with Na^+ only, with Na^+–Ca^{2+} and with Na^+–Mg^{2+} was determined (Fig. 3 *b*). In the buffer containing Na^+ as the only metal ion, the binding increased slightly from 19 to 23 % when the ionic concentration was increased from 0 to 25 mM, and remained at about 21 % through the range of ionic concentrations from 50 to 150 mM. In the Na^+–Ca^{2+}-containing buffer, when Ca^{2+} was present (10–150 mM), binding increased gradually from 19 to 32 %. However, in the Na^+–Mg^{2+}-containing buffer, binding was increased to 27 % at low Mg^{2+} concentrations (10–25 mM) and then decreased drastically at higher Mg^{2+} concentrations (50–150 mM).

Binding of AcMNPV BV to cells pretreated with enzymes

The binding analysis clearly showed specific binding sites on the surface of Sf21 cells (Fig. 1). The biochemical nature of the binding site was studied by treating the cell surface with proteases and glycosidases. Pretreatments of cells with *N*-glycosidase F, *O*-glycosidase and neuraminidase did not result in any significant changes in binding (Table 1). However, pretreatments of cells with proteases resulted in reduced binding. Treatments of cells with trypsin, pronase and dispase all reduced virus binding. For the three protease treatments, the extent of binding reduction was correlated with the con-

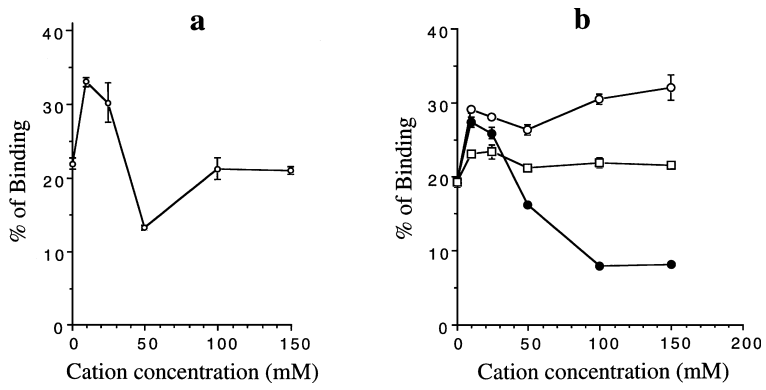

Fig. 3. Ionic effect on binding of AcMNPV BV to Sf21 cells. (*a*) Effect of cation strength on binding was determined in 20 mM BisTris–HCl buffer pH 6·2, using a series of concentrations of cations in a constant composition ratio (Na^+:Ca^{2+}:Mg^{2+} at 1:2:2). Error bars represent standard errors from triplicate samples. (*b*) Effect of Na^+, Ca^{2+} and Mg^{2+} cation strength on binding. The binding was determined in 20 mM BisTris–HCl buffer pH 6·2 with a series of concentrations of Na^+ (□), Na^+ and Ca^{2+} at a ratio of 1:4 (○) and Na^+ and Mg^{2+} at a ratio of 1:4 (●). Error bars represent standard errors from triplicate samples.

Table 1. Binding of AcMNPV BV to Sf21 cells pretreated with enzymes

Enzyme/enhancin	Concentration	Relative binding*(%)	Standard error*(%)
Glycosidases			
N-Glycosidase F†	0·5 U/ml	100·4	0·8
	1 U/ml	102·5	2·3
O-Glycosidase†	2·5 mU/ml	104·8	1·3
	5 U/ml	106·6	2·6
Neuraminidase†	0·1 U/ml	102·5	3·2
	1 U/ml	99·3	1·6
Proteases			
Trypsin‡	0·05 μg/ml	82·8	3·5
	0·1 μg/ml	75·1	2·6
Pronase‡	0·5 μg/ml	83·8	2·7
	1 μg/ml	69·7	0·9
Dispase‡	0·5 μg/ml	80·6	3·0
	1 μg/ml	69·0	2·7
Enhancin			
TnGV enhancin§	25 μg/ml	99·2	3·3
	50 μg/ml	95·1	1·6

* Relative binding is the percentage of binding compared with control. Binding values and standard errors were calculated from triplicate samples.
† Sf21 cells pretreated with a glycosidase at 28 °C for 1 h before binding assays were conducted.
‡ Sf21 cells pretreated with a protease at 37 °C for 20 min before binding assays were conducted.
§ Sf21 cells preincubated with TnGV enhancin for 2 h before binding assays were performed. Enhancin was also present when cells were incubated with the virus.

centration of proteases used (Table 1). The reduction of binding to Sf21 cells was not as great as that reported by Wickham *et al*. (1992). The attachment of Sf21 cells to a culture plate was much more susceptible to protease treatment, so in order not to distort the Sf21 monolayer the protease concentrations used in this study were 1000-fold or more lower than those used for *T. ni* cells (Wickham *et al*., 1992). In addition, the incubation period with proteases was shorter.

Binding of AcMNPV BV to Sf21 cells in the presence of TnGV enhancin

Tanada (1985) hypothesized that enhancins may facilitate baculovirus infections by mediating virus binding as an 'attachment molecule'. To test this hypothesis, binding of AcMNPV BV to Sf21 cells, pretreated with and in the presence of TnGV enhancin, was determined and compared with the enhancin-minus control treatment. The presence of 25 and 50 μg/ml enhancin in the medium did not result in any increased binding (Table 1).

Visualization of virus entry and membrane fusion

R18 is a fluorescent membrane probe inserted into the virus envelope, thus allowing the location of the virus envelope to be tracked. After a 1–2 h incubation of Sf21 cells with R18-labelled virus at 4 °C, virus particles were bound to the cell surface. When free (unbound) virus particles were removed and the cells incubated at pH 6·2 or 5·0 at 28 °C, the presence of virus envelopes could be observed under a fluorescence microscope. At pH 6·2, the fluorescent particles (virions) were initially observed on the surface of the cell plasma membrane and subsequently seen to localize within the cytoplasm (Fig. 4*a–c*). After incubation for 20 min at 28 °C and pH 6·2, most of the bound virions were still at the cell surface (Fig. 4*a*). At 45–90 min, after the cells were switched to 28 °C, virions were observed inside the cytoplasm (Fig. 4*b*, *c*). This process, which took place within 90 min, is indicative of endocytosis. In contrast, at pH 5·0 the virus envelopes fused directly to the cell plasma membrane, resulting in fluorescing plasma membranes (Fig. 4*d–f*). When the cells were treated with sodium azide at pH 5·0, virus envelopes were fused to the cell plasma membrane (Fig. 5*c*, *d*). In addition to blocking endocytosis, no detectable fusion at the plasma membrane was observed at pH 6·2 even after a prolonged incubation (70 min) at 28 °C (Fig. 5*a*, *b*).

Fusion kinetics of virus envelopes to cell membranes

The fusion kinetics of virus envelopes to cell membranes were determined by measuring the increasing fluorescence intensity, which is the effect of dequenching of R18, resulting from membrane fusion (Hoestra *et al*., 1984). The kinetics of fusion showed that the fusion initiation rates at pH 5·0 and 5·5 (Fig. 6*a*) were much higher than those at pH 6·0–6·5 (Fig. 6*b*). The percentage of fusion at pH 5·0 and 5·5 (Fig. 6*a*) was also higher than that at pH 6·0–6·5 (Fig. 6*b*). These kinetics studies provide further evidence that membrane fusion is triggered by acidification.

To confirm that the increased fluorescence detected at pH 6·0–6·5 (Fig. 6*b*) was due to the fusion of virus envelopes to endocytic vesicles, the cells were treated with sodium azide (an endocytosis inhibitor) and chloroquine (a weak base inhibiting endocytic acidification). The fusion of virus envelope to cells treated with chloroquine was reduced more than 50% compared to control cells at pH 6·2. The fusion of virus envelopes with cells treated with sodium azide was near zero (Fig. 6*c*).

Fusion of AcMNPV BV to cells with TnGV enhancin

To test the possible effect of TnGV enhancin on membrane fusion, R18-labelled virus was bound to Sf21 cells, treated with the enhancin and assayed for fusion. The kinetics of fusion in the presence or absence of enhancin at both pH 6·3 and 5·5 did not show differences in fusion initiation rate or percentage of fusion under the prevailing conditions (Fig. 7).

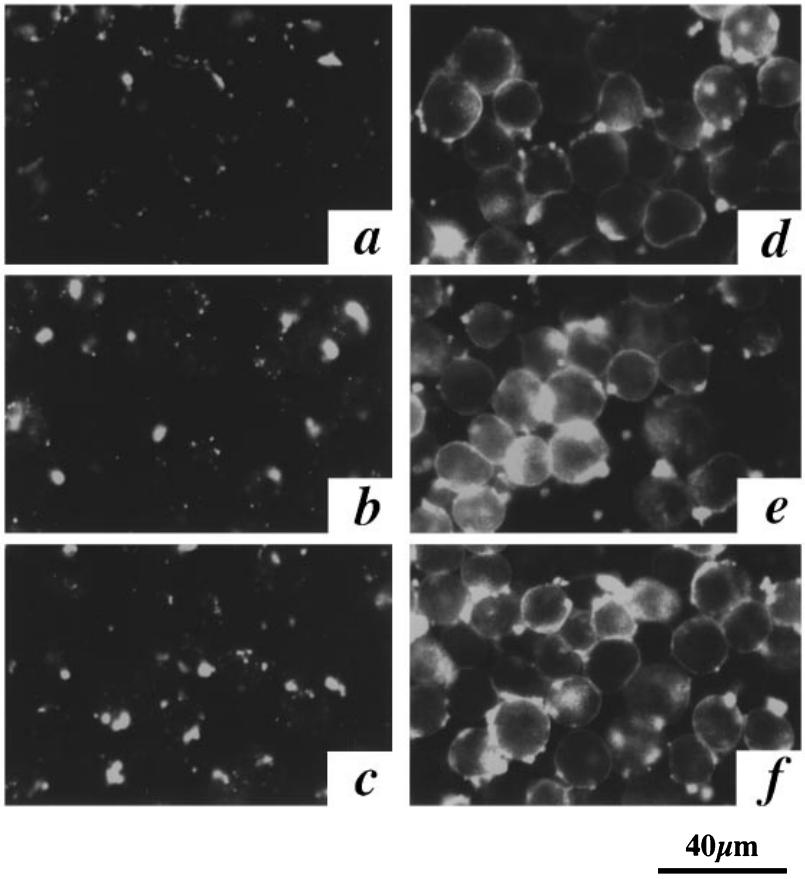

Fig. 4. Visualization of AcMNPV BV interacting with Sf21 cells at normal and acidic pH values. Sf21 cells were incubated with R18-labelled AcMNPV BV at 4 °C for 2 h. Unbound virions were removed by washing. The cells with R18-labelled AcMNPV BV were switched to 28 °C at normal pH (6·2) (*a-c*) or an acidic pH (5·0) (*d-f*). The localization of R18-labelled virus envelopes and their fusion to cell membranes was observed by fluorescence microscopy at 20 min (*a*, *d*); 45 min (*b*, *e*); and 90 min (*c*, *f*) after the cells were switched to 28 °C.

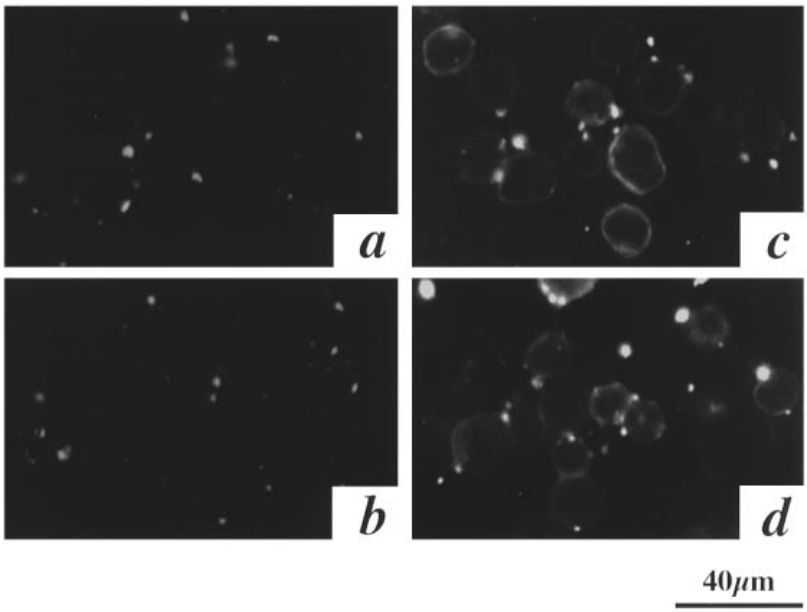

Fig. 5. Observations by fluorescence microscopy of AcMNPV BV interacting with Sf21 cells treated with sodium azide. Sf21 cells were treated with 0·1% sodium azide to abolish endocytosis and incubated with R18-labelled AcMNPV BV at 4 °C. Unbound virions were removed by washing and the cells were switched to 28 °C at pH 6·2 (*a*, *b*) and pH 5·0 (*c*, *d*). Fusion of the virus envelope to cell membranes was observed under a fluorescence microscope at 15 min (*a*, *c*) and 70 min (*b*, *d*) after the cells were switched to 28 °C.

Discussion

Although receptors for baculoviruses have not been identified on host plasma membranes, it is important to understand the binding characteristics of baculoviruses to insect cells. Fundamental knowledge of this mechanism could lead to the identification and characterization of cellular receptors for a variety of baculoviruses. Other than the study by Wickham *et al.* (1992), there have been no reports on the binding of baculovirus BV to insect cells. Proteinaceous specific

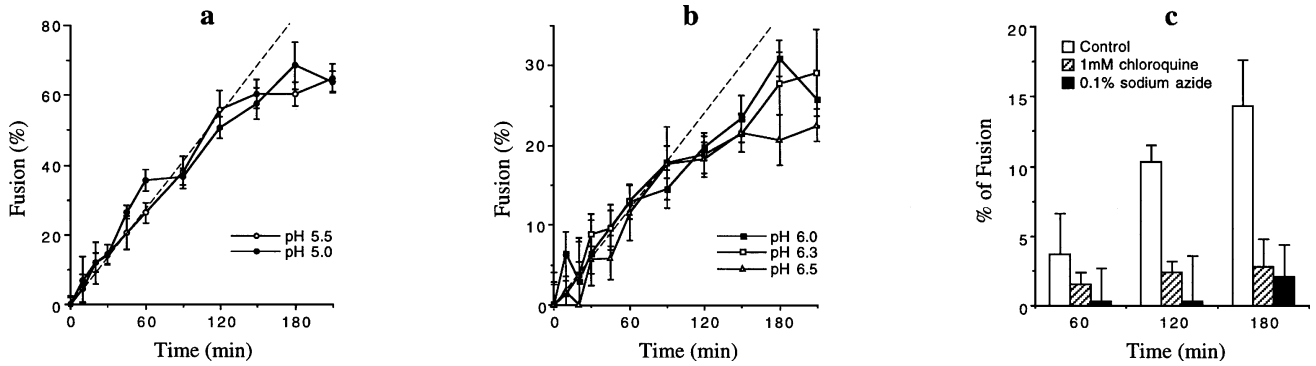

Fig. 6. (*a*, *b*) Fusion kinetics of AcMNPV BV envelope to Sf21 cells. Sf21 cells were incubated with R18-labelled AcMNPV BV at 4 °C. Unbound virions were removed from the cells by washing. The cells with bound R18-labelled virus were suspended in Grace's medium at pH 5·0 (●) to 5·5 (○) (*a*); and pH 6·0 (■), 6·3 (□) and 6·5 (△) (*b*) at 26 °C. Fluorescence intensities of the cell suspensions were measured from 0–3 h. Error bars represent standard error from triplicate measurements. Slopes of dashed lines represent fusion initiation rates at pH 5·0–5·5 and 6·0–6·5, respectively. (*c*) Fusion kinetics of AcMNPV BV envelope to Sf21 cells treated with sodium azide and chloroquine. Sf21 cells were incubated with R18-labelled AcMNPV BV at 4 °C. Unbound virions were removed by washing. Cells with bound R18-labelled virus were treated with 0·1% sodium azide (solid bars) and 1 mM chloroquine (shaded bars). A non-inhibitor-treated control was also included (open bars). Fusion kinetics were determined in Grace's medium (pH 6·2) at 26 °C. Error bars represent standard errors from triplicate measurements.

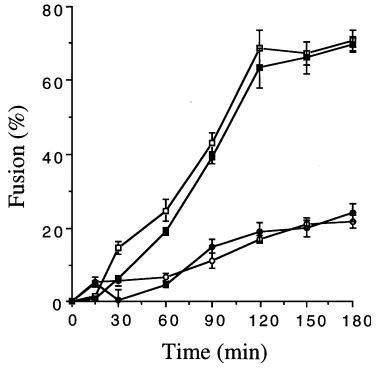

Fig. 7. Fusion kinetics of AcMNPV BV envelope to Sf21 cells in the presence or absence of TnGV enhancin. Sf21 cells were incubated with R18-labelled AcMNPV BV at 4 °C. Unbound virions were removed by washing. These cells were then treated with TnGV enhancin (50 μg/ml; ●, ■) and fusion kinetics were determined. Non-enhancin-treated controls are also shown (○, □). The fusion kinetics were determined at both normal cell culture pH (6·3; ○, ●) and acidic pH (5·5; □, ■). Error bars represent standard errors from triplicate measurements.

binding sites for AcMNPV BV on a *T. ni* cell line (BTI-Tn-5B1-4; Granados *et al.*, 1994) were reported previously (Wickham *et al.*, 1992). The binding of AcMNPV BV to Sf21 cells was also susceptible to proteases but not to glycosidases (Table 1), which was consistent with the report by Wickham *et al.* (1992). The affinity for the binding of AcMNPV BV to Sf21 cells ($2{\cdot}3 \times 10^{10}$ M^{-1}) (Fig. 1*b*) was similar to the affinity of binding to BTI-Tn-5B1-4 cells (Wickham *et al.*, 1992). When compared to the number of binding sites on BTI-Tn-5B1-4 cells ($6{\cdot}0 \times 10^3$ binding sites per cell; Wickham *et al.*, 1992), Sf21 cells have twofold fewer specific binding sites ($3{\cdot}1 \times 10^3$ binding sites per cell) for AcMNPV BV (Fig. 1*b*). Sf21 cells are approximately 50% smaller than BTI-Tn5B1-4 cells (Wickham, 1991); therefore for binding assays we used confluent monolayer Sf21 cells at a density of 6×10^5 cells per well, whereas Wickham *et al.* (1992) used BTI-Tn-5B1-4 cells at 4×10^5 cells per well. Consequently, the density of binding sites on Sf21 cells on a surface-area basis was approximately 80% of the density on BTI-Tn-5B1-4 cells. The binding of AcMNPV BV to Sf21 and BTI-Tn5B1-4 cells was remarkably similar, despite the fact that the lines were derived from different insect species. The number of specific binding sites for AcMNPV BV on these two cell lines is much less than the 10^6 specific binding sites per cell reported for the occluded virus phenotype (OV) of *Lymantria dispar* NPV (LdNPV) and its homologous cell line IPLB-LdEIta (Horton & Burand, 1993). Since the OV phenotype normally does not infect cultured cells, the significance of the OV binding may not be relevant to its biological function.

The range of pH used for the binding studies in this report represented physiological conditions for the cells where membrane fusion normally does not occur. Under these conditions the binding media and buffers were stable. The pH effect on virus binding varied depending on the medium used (Fig. 2). The binding of AcMNPV BV to Sf21 cells reached a maximum near pH 6·0 in sodium phosphate buffer and Rinaldini's solution and decreased with increasing pH values. In contrast, the binding in ExCell 400 medium reached a maximum at pH 6·5–6·8 and a minimum at pH 5·7. Clearly, the pH effect on virus binding is altered by media composition, although the mechanism of this effect is not known. The effect of medium pH on the AcMNPV BV binding in ExCell 400 medium is similar to the pH effect on attachment of TnNPV BV to *S. frugiperda* cells in a modified Grace's medium (Wang & Kelly, 1985). This pH effect on binding is also consistent with the pH effect on infectivity of AcMNPV BV in cell culture (Dougherty *et al.*, 1981).

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binding to cells (Lentz, 1990). The effect of cation strength on AcMNPV BV binding showed that with an increase in cation concentration, virus binding reached a maximum at 10 mM, but was reduced at higher cation concentrations (Fig. 3*a*). These findings suggested that a cation effect on binding is not a simple charge effect. Binding studies with combinations of ionic strength and composition (Fig. 3*b*) provided additional data on virus binding and showed that Na^+ did not have a significant effect on binding, and therefore electrostatic charge did not appear to be a major force in the binding of AcMNPV BV to Sf21 cells. In some instances, electrostatic forces may play a role in the binding of vertebrate viruses to cells; however, these forces result in nonspecific adherence of virions to the cell surface (Tardieu *et al.*, 1982). Increasing the concentration of Ca^{2+} increased virus binding. In contrast, at a low concentration the presence of Mg^{2+} increased binding; however, at high concentrations binding decreased. Therefore, bivalent cations appear to be involved in the binding of AcMNPV BV to Sf21 cells, although the mechanism(s) by which Ca^{2+} and Mg^{2+} are involved in virus binding is not understood.

In addition to the enhancement of OV infectivity in lepidopterous insect larvae by enhancins (Hukuhara *et al.*, 1987; Gallo *et al.*, 1991), many studies have reported that the enhancin from *Pseudaletia unipuncta* granulovirus (PuGV) can dramatically increase the infectivity of BV from several different baculoviruses, including AcMNPV, to insect cell lines derived from various species (see Corsaro *et al.* 1993 for review). Uchima *et al.* (1989) reported that a baculovirus enhancin from PuGV increased AcMNPV BV infection in Sf21 cells. The authors proposed that the enhancin protein was a 'binding molecule' and mediated binding of virus to cells resulting in a higher rate of infection. The enhancin from PuGV and that from TnGV are virtually identical in their amino acid sequences (over 98% identity; Roelvink *et al.*, 1995). However, the binding of AcMNPV BV to Sf21 cells, pretreated with and in the presence of TnGV enhancin up to 50 μg/ml (a dose sufficient for the enhancin from PuGV to give maximum enhancement in cell culture; Uchima *et al.*, 1989) did not show increased binding (Table 1). These results do not support the hypothesis proposed by Tanada (1985, 1989) and Uchima *et al.* (1989), although it could not be completely ruled out that slight differences in the amino acid sequence between TnGV and PuGV enhancin (Roelvink *et al.*, 1995) is significant enough to result in different effects on binding and fusion.

Adsorptive endocytosis has been proposed as the productive entry pathway for AcMNPV BV (Volkman, 1986). This pathway was further confirmed by acidic pH-induced syncytia formation of AcMNPV-infected cells (Leikina *et al.*, 1992) and acidic pH-induced syncytia formation of baculovirus envelope protein (gp64)-expressing cells (Blissard & Wenz, 1992). In contrast, direct fusion of TnNPV BV envelope to cell plasma membranes was also suggested to be a primary entry pathway, based on the results of ineffective blockage of virus infection (Wang & Kelly, 1985) and membrane fusion (Kozuma & Hukuhara, 1994) by endocytic acidification inhibitory reagents. In this paper, we have provided a means to trace the fate of the virus envelope in living cells by fluorescence microscopy, using virus labelled with R18. Fluorescence microscopy demonstrated that (*a*) no visible fusion of the virus envelope to cell plasma membrane occurred at normal pH after prolonged incubation (Figs 4 and 5); (*b*) virus particles were endocytosed at normal pH; and (*c*) the fusion of virus envelope to cell membrane was triggered by an acidic pH. These observations were consistent with earlier results of Volkman *et al.* (1986) and Charlton & Volkman (1993) and confirm the concept that endocytosis is the primary pathway for AcMNPV BV entry.

For membrane fusion kinetic studies (Fig. 6), cells were incubated with R18-labelled virus at 4 °C and, unlike other studies which examined baculovirus membrane fusion (Horton & Burand, 1993; Kozuma & Hukuhara, 1994), unbound virions were removed before membrane fusion assays were conducted. Therefore the binding and membrane fusion kinetics were separated and the effect of pH on binding was minimized. Membrane fusion kinetics confirmed our microscopic observations (Fig. 4) and, unlike the results of Kozuma & Hukuhara (1994), clearly showed that AcMNPV BV envelopes fused to cell membranes at acidic pH values (Fig. 6*a*). Although lower levels of increased fluorescence were also detected between pH 6·0 and 6·5 (physiological pH for cultured cells; Fig. 6*b*), we believe that the fusion detected at this pH range occurred mainly in endocytic vesicles, since at this pH range fusion was blocked by chloroquine and sodium azide (Figs 5 and 6*c*). The binding studies, combined with both the fluorescence microscopy and membrane fusion kinetics reported in this paper, further substantiate the suggestion that endocytosis is the primary mechanism for baculovirus BV entry into cells *in vitro*. Under the conditions of the studies reported here, TnGV enhancin did not show any significant effect on either virus binding or fusion to cultured cells (Table 1; Fig. 7), suggesting that the primary mode of action of enhancin is at the insect midgut level (Wang *et al.*, 1994).

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