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Herpes simplex virus type 1 penetration initiates mobilization of cell surface proteins

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Changes in membrane structure resulting from herpes simplex virus 1 (HSV-1) penetration were detected using fluorescence photobleaching recovery methods. The effect could be blocked by inhibitors of viral and cellular processes involved in virus penetration. A rapid mode of HSV-1 strain KOS penetration into VERO cells at 37 °C normally occurs after a 5 min lag period and is 90–95% complete within 20–30 min. Rates of cell surface protein diffusion increase 2–3-fold after 5 min and return to normal after 25–30 min, this return correlating temporally with the penetration of the virus. At pH 6.3 the lag period preceeding penetration of HSV is increased to 20 min and penetration proceeds much more slowly than at pH 7.4. Inhibition of virus penetration with cytochalasin B or with the antiherpes drug tromantadine also prevents the HSV-1-induced increase in cell surface protein mobility. Colchicine, which does not block HSV-1 penetration, prevents the recovery of the membrane following virus penetration. Therefore, the changes in membrane structure characterized by increased cell surface protein mobility seem to be caused by virus penetration. Cytoskeletal function and integrity are required for the initiation of, and cell recovery from, virus penetration. A pH-sensitive activity, likely to be a virion fusion glycoprotein, is also required.

Introduction

The two major modes of viral penetration of host cells which have been described for enveloped viruses are receptor-mediated endocytosis and fusion with the host plasma membrane. Entry by receptor-mediated endocytosis utilizes the cellular mechanism for ligand uptake whereas fusion involves insertion of the viral envelope into the plasma membrane. The predominant pathway used by the virus seems to depend on the optimum pH for function of the protein(s) that facilitate fusion of the host and virus membranes [1]. Viruses internalized by receptor-mediated endocytosis have a low optimum pH for fusion and penetrate into the cell upon acidification of the virion-containing receptosome [2]. Viruses which fuse at the plasma membrane initiate the process at neutral pH.

HSV penetration by both mechanisms has been observed by electron microscopy [3–7]. However, a number of observations suggest that fusion of

Abbreviations: FPR, fluorescence photobleaching recovery; MOI, multiplicity of infection; PBS, phosphate-buffered saline; FBS, fetal bovine serum; S Con A, succinyl concanavalin A; herpes simplex virus, HSV.

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HSV occurs in viable cells: (1) Viral antigens appear on the cell surface immediately following infection [8]; (2) HSV immobilized to *Staphylococcus aureus* through antibody linkages binds and infects cells leaving the bacteria bound to the cell surface [9]; and (3) Fluorescence energy transfer from fluorescein-octyldecylamine-labeled virus to rhodamine-octyldecylamine-labeled cell membranes occurs upon infection [10]. Our recent studies have shown that penetration via fusion predominates; but, upon analysis of individual virus penetration, endocytosis of virus is also seen to occur though at a much slower rate (preliminary studies).

Penetration of enveloped viruses by fusion has been studied most extensively with Sendai virus. Sendai virus has the capacity to fuse with its target cell and can also cause cell-cell fusion immediately after viral penetration and the delivery of its genome to the cytoplasm [11,12]. Fusion of the virus is mediated by its F glycoprotein which has a neutral to basic optimum pH for fusion [13,14]. The attachment of the virus and its fusion to the cell membrane are accompanied by changes in membrane structure [15] which include fluidization of membrane lipids [16–18], increased mobility of cell surface proteins [19], intramembrane particle aggregation [20], calcium ion flux [21], and changes in membrane potential [22].

HSV-1 penetration is less well understood. The process is inhibited by low temperature [23], by disruption of microfilaments via cytochalasin B and cytochalasin D [24], by mildly acidic pH (preliminary studies) and by the antiviral drug tromantadine [25]. Studies with temperature-sensitive mutants suggest that the gB glycoprotein is likely to be the fusion protein for HSV [26] but other viral glycoproteins are also involved in the process.

Changes in cytoskeletal structure and function upon attachment and penetration of HSV-1 strain KOS to HEp-2 cells have been inferred from fluorescence photobleaching recovery (FPR) measurements of the lateral diffusion of succinyl-concanavalin A (S Con A)-labeled cell surface protein [27]. FPR is a laser optical technique which measures the lateral diffusion of fluorescently-labeled probes on the cell surface. Attachment of the virus to the cells caused a rapid reduction in protein lateral diffusion which was followed by a transient 2–3-fold increase in cell surface protein lateral diffusion. This lasted 20–30 minutes, the time required for completion of HSV penetration. In this study, the HSV-induced increase in cell surface protein mobility and its relation to virus penetration and microtubule and microfilament function were investigated using inhibitors of HSV penetration.

Materials and Methods

Buffers. Phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.4). Tris-buffered saline (TBS) contained 25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.7 mM NaH_2PO_4 (pH 7.4).

VERO cell and virus preparation. VERO (African green monkey kidney cell line) cells were provided by Dr. Carol Blair at Colorado State University and maintained in Eagle's modified minimal essential medium (Auto Pow, Flow Laboratories supplemented with 10% fetal bovine serum (FBS; K.C. Biologicals, Kansas City, MO)), gentamycin (50 μg/ml), L-glutamine (0.03%) and non-essential amino acids. Stocks of HSV-1 strain KOS (originally provided by Dr. Priscilla Schaffer) were prepared in VERO cells and titered by plaque assay [28].

Fluorescence photobleaching recovery measurements. VERO cells were grown on 10 mm cover slips to subconfluent monolayers under normal growth conditions. The coverslips were washed in PBS at 4° C and incubated with HSV-1 for 2 h at 4° C with enough virus to yield a multiplicity of infection (MOI) of less than 10. Incubation of HSV-1 with cells at 4° C prevents penetration of the virus into the cells, maximizes binding of the virus to the cells, and allows synchronization of subsequent steps in penetration [23,24]. The cells were then washed free of unbound virus. Cell surface proteins were labeled with 20 μg/ml TRITC-derivatized succinyl-Con A (S Con A; Vector Laboratories; Burlingame, CA) in 100 μl PBS for 20 min at 4° C. The concentrations of the probe are sufficiently low so as to have no observable effect on the system. The S Con A was added after the virus to minimize any interference

with binding of the virus to the cell. The coverslips were then washed with PBS, treated with the appropriate inhibitor for 5 min, if applicable, and transferred to the thermal stage of the Zeiss Universal fluorescence microscope maintained at 37°C. The photobleaching apparatus used in these experiments has been previously described [29]. To measure the lateral diffusion of TRITC-S Con A, a single photobleaching experiment consisted of 16 fluorescence measurements at 200 ms per point to establish the level of prebleach fluorescence, a 250 ms bleaching pulse, and 240 additional 200 ms measurements to delineate fluorescence recovery kinetics. Each photobleaching experiment was repeated at most three times at the same site. Data are presented as the average of lateral diffusion measurements performed at each condition taken within a 5 min time period. The time required to microscopically identify a cell and initiate the computer-controlled FPR experiment did not permit us to make measurements of protein lateral diffusion sooner than 2 min after the temperature shift.

Virus penetration assay. The time-course of HSV penetration was measured by protection from acid inactivation of extracellular virus using a modified protocol described by Huang and Wagner [23] and by Rosenthal et al. [27]. HSV-1 was added to VERO cells at 4°C in PBS (pH 7.4) and incubated for 5 min. The cells were then treated as described in the text of the paper and the temperature shifted rapidly to 37°C. This was designated as time zero. At different times after temperature shift. PBS adjusted to pH 3.0 was added to the cells for 1 min to inactivate extracellular virus. The acid was neutralized by washing with PBS and medium and the cells prepared for plaque assay. These experiments require a completely confluent monolayer and unlike HEp-2 cells used in other experiments, some of the cells in a VERO cell monolayer detach at 4°C. Thus, attachment and penetration are not distinguished using this procedure while only penetration of the virus was assayed during the FPR experiments.

Results

Penetration of HSV-1 strain KOS into various cells in a population can be synchronized by temperature shift from 4°C to 37°C. Treatment of extracellular virus with pH 3 buffer disrupts the virus. Internalization of the virus protects the virus from acid inactivation and can be assayed by plaque production [23]. Using this technique, it has been shown that HSV-1 strain KOS penetration into HEp-2 cells begins following a 5 min lag period and proceeds rapidly for 20–30 min at which time 90–95% of the innoculum is internalized [24,27]. Similar results are obtained for HSV-1 penetration into VERO cells (see below).

The effect of HSV penetration on the lateral diffusion of S Con A receptors is shown in Fig. 1. VERO cells were infected with virus or mock-infected for 2 h at 4°C and labeled with TRITC-S Con A for 20 min prior to transfer to the 37°C microscope stage of the FPR apparatus. The diffusion coefficient for TRITC-S Con A on sham-infected VERO cells averaged $2.7 \cdot 10^{-11}$ $cm^2 \cdot s^{-1}$. In virus-treated cells, the diffusion coefficient for mobile S Con A-labeled membrane proteins increased approximately 3-fold within 5–9 min following the temperature shift. This increase in protein lateral diffusion is concurrent with the time of initial virus penetration. The rate of cell surface protein diffusion returned to control levels after 25 min. This time course for increases in cell surface protein lateral diffusion is similar to that

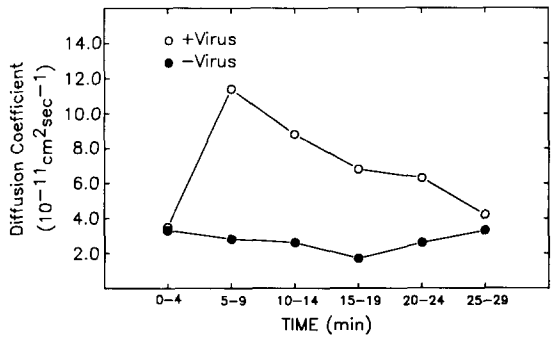

Fig. 1. The effect of HSV-1 penetration on cell surface protein mobility. VERO cells grown on glass cover slips were mock-infected (●) or infected (○) with HSV-1 strain KOS at 4°C for 1 h, unbound virus washed away, the cells labeled with TRITC-S Con A for 20 min at 4°C, washed, and equilibrated at 37°C on the microscope stage. Cells were viewed and fluorescence photobleaching recovery (FPR) measurements made within 2 min of temperature shift on randomly-chosen cells.

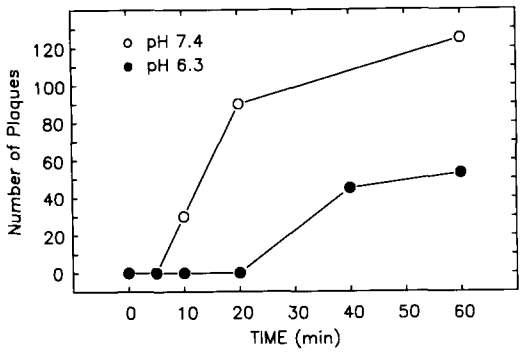

Fig. 2. Mild acidic pH inhibition of HSV-1 penetration into VERO cells. HSV-1 was added to VERO cells at 4° C for 5 min at pH 6.3 or 7.4, the temperature shifted to 37° C and extracellular virus inactivated at different times with pH 3.0 buffer. After 30 min at 37° C, one monolayer at each pH was washed and incubated for an additional 30 min at pH 7.4 at 37° C to indicate the reversibility of inhibition.

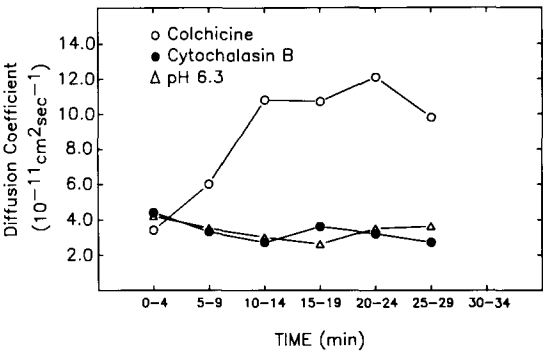

Fig. 3. The effect of penetration inhibitors on HSV-1-induced changes in cell surface protein mobility. Cells were prepared and FPR readings were performed as described for Fig. 1 except that cells were treated with the indicated inhibitors 20 min prior to temperature shift.

observed following HSV-1 infection of HEp-2 cells [27].

Recent studies have indicated that HSV-1 strain KOS penetration into HEp-2 cells is reversibly inhibited by exposure to pH below 7.0. This is also the case for VERO cells. Penetration of the virus proceded rapidly following a 5 min lag period at pH 7.4. However, penetration into cells maintained at pH 6.3 followed a 20 min lag period and proceeded at a much slower rate (Fig. 2). There was an equivalent recovery of input virus from samples at pH 6.3 and 7.4 after washing and incubation for an additional 30 min at pH 7.4 indicating that only the penetration of the virus was inhibited at the lower pH. Similarly, the HSV-induced cell surface protein mobility increase was prevented by incubation at pH 6.3 (Fig. 3) with no change in TRITC-S Con A lateral diffusion in mock-infected cells.

Penetration of HSV-1 can be reversibly inhibited by treatment of cells preincubated at 4° C with cytochalasins B or D [24]. Both drugs inhibit microfilament function [30,31]. Treatment of the cells with cytochalasin B (40 μg/ml) prevented the increase in protein lateral diffusion induced by HSV-1 infection (Fig. 3). Cytochalasin treatment of the mock-infected cells had no effect. Colchicine (40 μg/ml), an inhibitor of microtubule function, enhanced and lengthened the time period for the HSV-induced increases in cell surface protein lateral diffusion. Colchicine has no effect on HSV-1 penetration at sub-toxic concentrations [24].

The increase in cell surface protein mobility following virus infection was also inhibited by tromantadine (Fig. 4). Tromantadine is an antiviral drug effective against HSV and paramyxoviruses which inhibits an early step in infection [25,32]. The drug had no effect on cell surface protein lateral diffusion in the mock infected cells. Treatment of the infected cells with tromantadine caused a concentration-dependent inhibition of the virus-induced increases in protein lateral diffusion. In cells treated with tromantadine at a concentration of 10 μg/ml, the appearance of in-

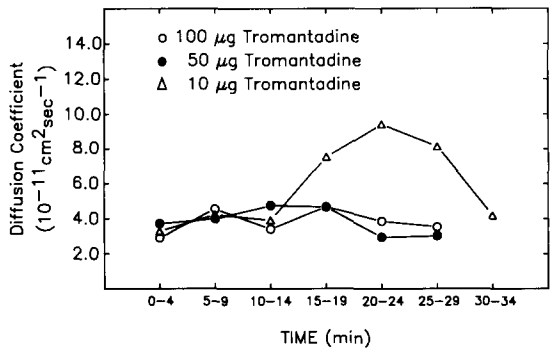

Fig. 4. Tromantadine effects on HSV-1 induced changes in cell surface protein mobility. Cells were prepared and FPR measurements were performed as described for Fig. 1 except that cells were treated with the indicated tromantadine concentrations 20 min prior to temperature shift.

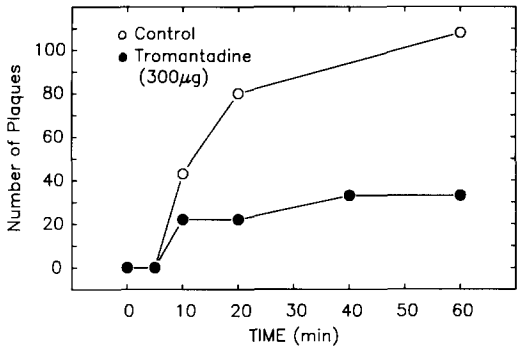

Fig. 5. Tromantadine inhibition of HSV-1 penetration into VERO cells. HSV-1 and tromantadine (300 μg/ml final concentration) were added to VERO cells at 4°C for 5 min, the temperature shifted to 37°C and extracellular virus inactivated at different times with pH 3.0 buffer.

creased protein lateral diffusion was delayed. At the higher doses, no change in cell surface protein mobility was observed within the assay time period. This is a further indication that tromantadine inhibits an HSV penetration-related event. This is in agreement with the results shown in Fig. 5 which indicate that tromantadine inhibits or delays HSV uptake into VERO cells.

Discussion

The attachment and penetration of HSV-1 to HEp-2 and VERO cells initiates changes in the rate of cell surface protein lateral diffusion. Immediately following infection of HEp-2 cells with HSV-1, protein mobility is restricted as indicated by a decrease in the diffusion coefficient of TRITC-labeled S Con A receptors [27]. Concurrent with HSV-1 penetration into HEp-2 and VERO cells, a 2–3-fold increase in protein lateral diffusion occurs. After 90–95% of the innoculum has entered the cell, the diffusion coefficient of the cell surface protein returns to normal. Distinguishing the cellular effects of virus attachment and penetration is experimentally difficult. However, the conditions used in these studies allowed us to temporally separate these events. This is possible because the time course for attachment and penetration of HSV-1 is known [24,27] and the initiation of penetration can be synchronized. In order to show that the changes in protein lateral diffusion are associated with attachment and penetration, FPR measurements were made within the time frame required for attachment and penetration under physiological temperature and buffer conditions.

The predominant mode of HSV-1 penetration into HEp-2 and VERO cells is inhibited by mildly acidic pH as was the increase in cell surface protein mobility. At pH 6.3 initiation of virus penetration is retarded by 15 min and proceeds at a much slower rate than at physiological pH. The mechanism by which HSV-1 penetration is inhibited at the lower pH is not known. However, the activities of fusion proteins of other enveloped viruses are very sensitive to pH [2,13,33]. At mild acidic pH, it is likely that the activity of a fusion protein is reduced and that the fusion mode of virus entry is no longer available to HSV. Although it is possible for the virus penetration to occur through an alternate pathway under these restrictive conditions, no change in cell surface protein mobility was observed. Either this alternate pathway does not elicit a change in cell surface protein mobility or its longer lag period prevents measurement of the change within the interrogation period.

The use of HSV penetration inhibitors indicates that the increase in cell surface protein mobility observed by FPR is intimately associated with, and probably caused by, the penetration of the virus. Inhibition by treatment with cytochalasin of both virus penetration and the associated transient increase in protein lateral diffusion indicates that structural components containing intact microfilaments are needed for uptake of HSV-1. We have found that cytochalasins are active only if added to cells maintained at 4°C [24]. Microfilaments are dissociated in the cold [31] thus facilitating cytochalasin binding. Cytochalasin inhibition of HSV penetration might result from inhibition of a contractile process or, alternatively, from a block in reestablishment of microtubule-microfilament interactions required for penetration. Cytochalasins do not have direct activity on microtubules but can inhibit microtubule-dependent movement of vesicles in neurites [34], possibly through the action of microtubule-associated protein MAP-2 or a similar protein. Microtubule activity may not be directly involved in HSV penetration but is required for membrane recovery from virus

penetration. Colchicine inhibition of microtubule function prevented or slowed the recovery of the cell following the virus induced increase in cell surface protein mobility.

Tromantadine treatment also inhibited the change in cell surface protein mobility increase upon penetration. The mode of action of tromantadine is not known. Drug treatment delays penetration of HSV-1 strain KOS into VERO and HEp-2 (preliminary studies) cells at suboptimal concentrations and prevents penetration at higher concentrations (Fig. 5). This same concentration dependence was observed for the protein lateral diffusion change upon penetration. Effects of tromantadine on cytoskeletal structures have not been reported.

Changes in membrane structure upon virus infection have been observed by Lyles and Landsberger [17,18]. Their ESR studies indicate that changes in lipid fluidity occur upon multivalent ligand attachment (Sendai virus, influenza virus, wheat germ agglutinin and concanavalin A) to avian, but not human, erythrocytes. Avian erythrocytes have microtubules and microfilaments while human erythrocytes do not. The change in lipid fluidity was inhibited by treatment with colchicine, vinblastine and tetracaine but enhanced by cytochalasin B. They suggested that protein mobility would be inversely affected. Their experiments were performed under conditions in which the ligand would bind to, but not penetrate, the cell.

Maeda et al. [19] have observed 2–3-fold increases in protein mobility and a decrease in lipid mobility following Sendai virus infection of human KB and mouse 3T3 cells at high multiplicity of infection (MOI). These changes could be attributed to the presence of a functional F protein. However, since the FPR measurements were taken after 30 min incubation with virus at 4°C followed by 60–75 minutes at 37°C, it is unlikely that an effect of virus penetration is being observed. In contrast, Henis et al. [35] and Aroeti and Henis [36] investigated changes in cell surface protein mobility following Sendai virus infection under very different conditions and reported that the virus did not change cell surface protein mobility. However, they measured protein lateral diffusion in the infected cells 90 min after infection and at room temperature. Temperatures below 37°C are not optimal for virus penetration. This may alter the observed cellular response and prevent detection of changes in cell surface protein lateral diffusion. The time scale used to examine protein lateral diffusion is also critical. HSV-induced changes in lateral diffusion are transient and FPR examination of the cells 30–40 min following infection would not reveal these increases in protein lateral mobility.

Our results from this present work and from other studies suggest the following model for HSV penetration. Multivalent binding of HSV-1 to specific target cell surface receptors initiates global protein anchoring to cytoskeletal components which is accompanied by a membrane potential change. Both of these activities trigger a microfilament contractile activity which facilitates the interaction of the viral envelope and plasma membrane. Fusion of the two membranes is then initiated by a pH-dependent activity, probably conferred by the gB protein. Fusion of the membranes initiates disruption of cytoskeletal structure as a consequence of either ion flux or physical expansion of the membrane surface area. The membrane then 'snaps back' to normal through the action of microtubules after completion of virus penetration.

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