NOTES

Herpes Simplex Virus Binding and Entry Modulate Cell Surface Protein Mobility

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Fluorescence photobleaching recovery measurements showed that herpes simplex virus type 1 attachment to target cells rapidly induced an anchorage modulation of cell surface protein mobility, an activity mediated by the cytoskeleton and associated with the multivalent attachment of other ligands (e.g., cells, lectins, or anti-immunoglobulin) to cell surfaces. The restriction in cell surface protein mobility was released concurrently with virus penetration. The effects of attachment and penetration on cell surface protein mobility and cytoskeletal function are some of the earliest cellular changes induced by herpes simplex virus infection.

To infect a cell, herpes simplex virus (HSV) must first recognize and bind to a receptor on the cell surface and then penetrate the plasma membrane. These activities require both viral and cellular functions (6, 10). The viral glycoproteins have been shown to be important for recognition and entry of HSV (1, 16, 19); however, the mechanisms for virus attachment and internalization are less well understood (6, 10). It has been presumed for HSV and other enveloped viruses that viral glycoproteins recognize and bind to specific receptors on target cells (6), establishing an initial weak interaction. Subsequently, a strong, irreversible bond is formed by many viral glycoprotein-cell surface receptor interactions (10). Penetration of HSV into the cell then follows by either fusion of its envelope with the plasma membrane or viropexis (1, 8, 10, 18).

We report here on the effects of HSV type 1 (HSV-1) infection on the cell surface protein (CSP) mobility of HEP-2 cells and correlate the kinetics of CSP mobility changes with the kinetics of virus attachment and penetration. Many CSPs interact with the underlying cytoskeleton, which modulates their intramembrane movement. Multivalent attachment of other ligands, such as lectins or anti-immunoglobulin, to the cell surface has been associated with a restriction in the lateral mobility of surface proteins, including those distal to the bound ligand (5). This phenomenon, termed anchorage modulation, is apparently mediated by both microfilaments and microtubules.

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For our investigation, we used the optical technique of fluorescence photobleaching recovery (FPR) to measure changes in the mobility of fluorescently labeled proteins on the cell surface. In this technique, a brief pulse of high-intensity laser light is used to irreversibly bleach a small area of the cell surface. The kinetics of recovery of fluorescence in this area yield a diffusion constant for the labeled cell

surface proteins (12). FPR measurements were made on a Zeiss Universal microscope equipped with a heated thermal stage and modified for FPR measurements as described previously (12).

HEP-2 cells were maintained in Dulbecco modified minimal essential medium (Auto-Pow; Flow Laboratories, Inc.) supplemented with 10% newborn calf serum. Extracellular HSV-1, strain KOS, was collected from spent media of HEP-2 cell cultures infected at a low multiplicity of infection. Infectious virus was quantitated by plaque assay (3). Enveloped particle counts were determined by electron microscopy (13). The virus stock utilized for all experiments described herein had an approximate particle-to-PFU ratio of 10. The multiplicity of infection was calculated from PFU measurements. For FPR measurements, HEP-2 cells grown to subconfluent monolayers on cover slips were incubated at 4°C with 0.2 µg of tetramethyl rhodamine isothiocyanate-conjugated succinyl concanavalin A (TRITC-SConA) (Vector Laboratories Inc.) in Dulbecco phosphate-buffered saline (PBS) for 10 min. Unbound conjugate was washed away with PBS. TRITC-SConA nonspecifically labels mannose-containing glycoproteins and glycolipids but, unlike concanavalin A, does not induce receptor capping or patching (11). Cells were viewed through a 50× Leitz water immersion lens focused through a drop of PBS on the cells. Under such conditions, cells maintained >85% viability for the 30-min time course of an experiment (as determined by cell morphology and trypan blue exclusion). The cells and the location on the cells for FPR analysis were chosen at random. The fluorescence recovery data were processed on-line by a NOVA 3/12 computer (12) to obtain the percent fluorescence recovery and average diffusion coefficient. Results presented herein are representative of more than three runs for each experiment.

Figure 1A shows the effects of HSV on the mobility of cell surface proteins. Control readings were taken before the addition of virus. HSV-1 or medium was then added to the cells, and data collection was continued. CSP mobility, measured before the addition of virus or on cells mock

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FIG. 1. FPR measurements. (A) Direct addition of virus. HEP-2 cells grown on glass cover slips were labeled with TRITC-SConA, washed, and equilibrated at 37°C in PBS on the microscope stage. Cells were viewed and FPR measurements made on randomly chosen cells through a water immersion lens focused through a 100- μl drop of PBS. After initial fluorescence measurements, a medium control (shaded area) or HSV-1 (3×10^4 PFU/10 μl ; multiplicity of infection, ca. 2) was added directly to the PBS, and measurements were taken on randomly chosen cells. (B) HSV prebound to cells at 4°C, followed by temperature shift. HEP-2 cells were incubated with 3×10^4 PFU of HSV for 15 h, washed with PBS at 4°C, labeled with TRITC-SConA at 4°C, washed again, and then rapidly equilibrated at 37°C on the microscope stage. FPR measurements were begun within 1 min of temperature shift. Shaded area represents readings of mock-infected cells incubated at 4°C for 15 h, labeled

infected with medium, stabilized rapidly upon incubation at 37°C at a mean ($\pm$ standard deviation) value of $1.2 \times 10^{-10} \pm 0.2 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ (Fig. 1A, shaded area). This value is typical for the mobility of succinyl concanavalin A-labeled CSP on a variety of cell types (14). Upon the addition of 3×10^4 PFU of HSV to the HEP-2 cells (multiplicity of infection, ca. 2), an immediate threefold reduction in CSP mobility was observed. The reduction in CSP mobility lasted for 6 to 8 min, followed by a transient fluidization and then a return to control levels ca. 13 min after the addition of virus. Fluorescence recovery after photobleaching averaged about 50%, independent of the presence or absence of virus. A similar degree of fluorescence recovery, independent of the extent of ligand binding to concanavalin A-conjugated platelets, was obtained by Schlessinger et al. (17).

The effect of virus attachment on CSP mobility, independent of the effects of virus entry, was studied on HEP-2 cells infected with HSV at 4°C. Addition of HSV to cells at 4°C prevents entry of virus into the cell, thus limiting the virus to attachment on the outside of the cell (7). This procedure maximizes input virus binding, synchronizes subsequent steps in viral infection, and minimizes potential competition for receptor binding between the virus and TRITC-S ConA. Unlabeled HEP-2 cells were incubated at 4°C with 3×10^4 PFU of HSV or PBS for 15 h, washed at 4°C to remove unbound virus, labeled with TRITC-SConA, washed again, and then rapidly equilibrated at 37°C on the thermal stage of the microscope for FPR measurements.

As in the previous experiments, cell surface-bound virus restricted CSP mobility with a decrease in average diffusion coefficient from $1.8 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ for control cells to $0.42 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ within 2 min of the temperature shift (Fig. 1B). CSP mobility remained lowered for 8 to 10 min, followed by a transient increase in CSP mobility with a return to control levels ca. 20 min after the temperature shift. The effects of HSV on CSP mobility are similar for cells infected at 37 or 4°C and then shifted to 37°C. Hence, the restriction in CSP mobility is most likely due to HSV attachment to the HEP-2 cells. Differences in the kinetics of recovery for the experiments shown in Fig. 1A and B may be due to differences in experimental conditions, e.g., incubation of target cells overnight at 4°C.

To show that FPR measurements were being made at locations away from the site of virus attachment, we developed a means of microscopically visualizing HSV binding to cells (C. M. Hodnichak, E. Turley-Shoger, J. G. Mohanty, and K. S. Rosenthal, J. Virol. Methods, in press). By use of rabbit HSV antibody, HSV was attached to a morphological tag, formalinized *Staphylococcus aureus* strain Cowan A (IgG-sorb) (New England Enzyme Center) (9). HSV-1 was linked to IgG-sorb (9) by rabbit antibody prepared against Triton X-100 extracts of HSV-1 strain KOS (characterized by immunofluorescence and neutralization). Equal volumes of a 10% IgG-sorb suspension and antisera were incubated for 20 min, washed eight times to remove unbound antibody, and incubated with dextran T-10 density gradient-purified (2), concentrated HSV-1 strain KOS. The complex (St-Ab-HSV) was washed again eight times to remove unbound virus. Whereas the St-Ab-HSV complex bound to HEP-2

with TRITC-SConA, and analyzed as described for the other samples. Control-zero time (○). (C) St-Ab-HSV and FPR measurements. HSV was attached to an IgG-sorb-anti-HSV complex and washed to remove unbound virus as described in the text. HEP-2 cells were incubated with St-Ab-HSV complex or medium overnight at 4°C, and FPR measurements were made as described in (B).

cells, binding was not observed for IgG-sorb incubated with normal rabbit serum or without HSV. The virus in the St-Ab-HSV complex is still viable and can form infectious centers, but it is unlikely that this is due to free virus. The St-Ab-HSV complex was stable under incubation at 37°C for 16 h in medium with no shedding of infectious virus (data not shown). HEp-2 cells were incubated at 4°C with the St-Ab-HSV complex as described for free HSV and handled as described for the experiment shown in Fig. 1B. FPR measurements were made only on those cells which bound the St-Ab-HSV complex, and measurements were made on regions of the cell as distant from the aggregate as possible. Upon temperature shift, a biphasic CSP mobility response was observed that resembled that for free virus (Fig. 1C). Hence, HSV attachment affects CSP mobility at sites away from the virus binding site.

The immediate mobility reduction caused by virus binding was temporary. CSP mobility increased ca. 10 min after virus addition or temperature shift, the time period required for HSV penetration to occur (7). Figure 2 shows the kinetics of virus penetration as a function of the time required for HSV-1 strain KOS to become resistant to acid inactivation (7). Basically, HEp-2 cells grown in 24-well plastic plates (Costar), ca. 10^5 cells per well, were incubated with ca. 10^4 PFU of HSV for 4 h at 4°C, washed, and then incubated at 37°C for various times before the addition of 0.1 M glycine HCl (pH 3.0)-buffered saline. After 1 min, the acid was neutralized with medium and replaced with medium containing 1.5% methyl cellulose for plaque assay (3, 7). Penetration into the cell, as detected by protection from acid inactivation, occurred 6 to 8 min after a shift in temperature from 4 to 37°C. Virus penetration was completed within 20 min of the temperature shift.

These experiments indicate that attachment of HSV to the target cell surface induces a global decrease in CSP mobility similar to that caused by attachment of multivalent ligands to cell surface receptors (4, 5, 11, 12, 17). Restriction of CSP mobility presumably follows the cross-linking of a population of receptor proteins and the cytoskeletal elements with which they are associated. The rapid establishment of anchorage modulation upon either the addition of virus to TRITC-SConA-labeled cells or the shift to 37°C of cells preincubated with virus at 4°C makes it unlikely that the restriction in CSP mobility is an artifact of the conditions of virus addition, TRITC-SConA treatment, or temperature changes. In addition, the effects of the addition of the St-Ab-HSV complex to cells make it unlikely that FPR measurements were made on proteins other than TRITC-SConA-labeled cell surface proteins, e.g., on virus or viral glycoproteins. Anchorage modulation has been described by Henis and Elson (5) and by Gall and Edelman (4) when concanavalin A-conjugated platelets interact with lymphoid and epithelial cells, respectively. In both cases, attachment of the platelets reduced CSP mobility to an extent comparable to that observed here for HSV attachment to cells; however, no subsequent fluidization of CSP mobility was observed in either case.

An increase in CSP mobility greater than control levels followed the period of anchorage modulation in each of the HSV infection experiments. For the temperature shift experiments, the release of anchorage modulation occurred concomitantly with HSV penetration, and a return to control levels was observed upon completion of virus penetration. The extent of the change in CSP mobility that we observed is similar to that observed upon disruption of the cytoskeleton with cytochalasin and colchicine (5, 11), suggesting that a

FIG. 2. Kinetics of penetration of HSV. HEp-2 cells were incubated with ca. 10^4 PFU of HSV (●) for 4 h in PBS at 4°C, washed, and then incubated at 37°C for various times before the addition of 0.1 M glycine HCl (pH 3.0)-buffered saline. After 1 min, the acid was neutralized with medium, the medium was removed, and the cells were prepared for plaque assay (7). Alternatively, no acid treatment was used, but the cells were infected and assayed as described above (■). Infectious centers resulting from viable virus were quantitated after neutral red staining (3).

temporary disruption in the interaction of cytoskeleton and CSP accompany virus penetration. Potential mechanisms for the disruption of cytoskeletal assemblies by herpesvirus include a membrane potential change (15a); a change in intracellular Ca^{2+} , as observed upon Sendai virus fusion (20) and is known to affect cytoskeletal structure (15); or insertion of the viral envelope into the plasma membrane upon entry, separating previously contiguous CSPs.

Observation of anchorage modulation upon the HSV-target cell interaction is the first biochemical evidence that a multivalent attachment between virus and cell receptors precede virus penetration. The effects of attachment and penetration on CSP mobility and cytoskeletal function are some of the earliest cellular changes induced by a viral infection.

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