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Infection of polarized MDCK cells with herpes simplex virus 1: Two asymmetrically distributed cell receptors interact with different viral proteins

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ABSTRACT **Herpes simplex virus 1 attaches to at least two cell surface receptors. In polarized epithelial (Madin–Darby canine kidney; MDCK) cells one receptor is located in the apical surface and attachment to the cells requires the presence of glycoprotein C in the virus. The second receptor is located in the basal surface and does not require the presence of glycoprotein C. Exposure of MDCK cells at either the apical or basal surface to wild-type virus yields plaques and viral products whereas infection by a glycoprotein C-negative mutant yields identical results only after exposure of MDCK cells to virus at the basal surface. Multiple receptors for viral entry into cells expand the host range of the virus. The observation that glycoprotein C-negative mutants are infectious in many nonpolarized cell lines suggests that cells in culture may express more than one receptor and explains why genes that specify the viral proteins that recognize redundant receptors, like glycoprotein C, are expendable.**

Herpes simplex virus 1 (HSV-1) encodes at least eight proteins that have been shown to be glycosylated and components of the viral envelope (1). Of these glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, and gJ, the genes of five, gC, gE, gG, gI, and gJ, may be deleted from the viral genome without affecting the capacity of the virus to infect and produce infectious progeny in Vero and HEp2 cell lines (2–5). In this report we present definitive evidence that (*i*) HSV-1 encodes proteins that interact with at least two receptors, (*ii*) the two sets of receptors for HSV-1 in polarized Madin–Darby canine kidney (MDCK) cells are asymmetrically distributed on the cell surface, and (*iii*) infection of cells through one of the two receptors is dependent on the dispensable glycoprotein gC. To test the hypothesis that the dispensable glycoproteins interact with secondary cellular receptors, we chose a polarized cell line, because the spectrum of cells infected by HSV-1 in humans consists largely of polarized cells and it is in these cells that the role of the glycoproteins dispensable for viral infection in cells in culture might be apparent.

MATERIALS AND METHODS

Cells. Cell lines used were Vero, HEp-2, and MDCK cells from the American Type Culture Collection, squamous cell carcinoma (SCC13) cells from Elaine Fuchs (University of Chicago), and rabbit skin cells from J. McLaren (University of New Mexico). All cells were maintained in Dulbecco's modified Eagle's medium with 5% (vol/vol) fetal bovine serum. Cells used for infection were seeded on plastic 6-well dishes or on 24-mm collagen-coated Transwell-COL filters (Costar) at a density of 5×10^5 cells per well or filter and infected at least 24 hr after becoming confluent. Confluency of the monolayers was judged by light microscopic inspection.

Viruses. HSV-1(F) is a wild-type clinical isolate, passaged fewer than four times in culture (6). The isolation of HSV-1(MP) has been described (7).

RESULTS

Construction of Recombinant Viruses. The construction of the gC^- virus (Fig. 1) follows the outline for genetic engineering of sequence-specific deletion mutants reported elsewhere (9). Briefly, intact HSV-1(F) DNA was cotransfected with a plasmid carrying a gC gene from which were deleted the DNA sequences extending from 34 base pairs downstream of the methionine initiation codon in the mRNA to 195 base pairs upstream of the termination codon. Progeny of the transfection were plated on Vero cells, and several plaques were isolated that were unable to react with the anti-gC monoclonal antibody HC1 as described (10). These plaques were further purified, and the final virus stocks were tested for the ability to express gC on the cell surface and for the absence in the viral genome of the sequences deleted in the plasmid. One gC^- recombinant shown to lack the coding domain of gC was designated as R6012. This recombinant infected and replicated in all continuous nonpolarized cell lines tested (Vero, HEp-2, rabbit skin, and SCC13 epithelial cell lines). Two recombinant viruses in which the deletion was repaired, R6016 and R6017, were independently constructed by cotransfection of R6012 viral DNA with a plasmid containing an intact gC gene, followed by isolation and purification of plaques that were able to react with antibody HC1.

Infection of Polarized MDCK Cells. MDCK cells are polarized epithelial cells that sort and secrete a number of proteins in a directed manner, either to the apical or basal surfaces (11). One such protein is a heparin sulfate proteoglycan, a major constituent of the basal substratum of epithelial cells. This protein, which plays a major role in the attachment of HSV-1 to most cells in culture (12), has been reported to be secreted primarily on the basal surface of polarized MDCK cells (13). The polarized cells were grown either on plastic surfaces or on collagen-coated microporous membranes. On plastic surfaces, only the apical surface of MDCK cells is in contact with the medium. The microporous membrane filters may be placed in chambers designed to allow separation of medium in contact with the apical and basal surfaces. The studies that were done and the experimental results may be summarized as follows.

Wild-Type Virus, but Not R6012, Infected, Replicated in, and Formed Plaques in MDCK Cells Grown on Plastic Surfaces. Confluent monolayers of Vero or MDCK cells were infected with serial dilutions of each virus, fixed 48 hr after

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Abbreviations: HSV, herpes simplex virus; pfu, plaque-forming unit(s).

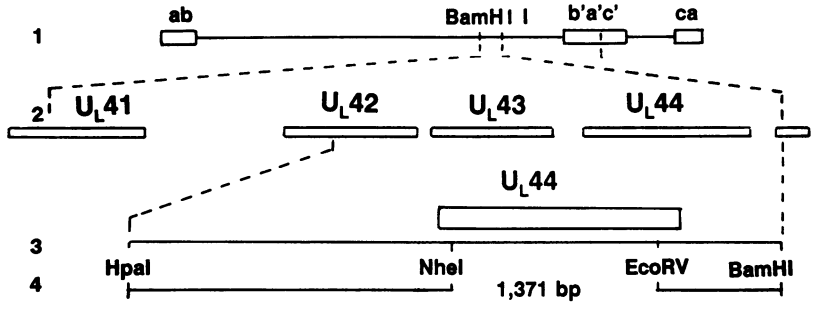

FIG. 1. Schematic diagram of HSV-1(F) and R6012 genomes. Line 1 shows the genomic arrangement of HSV-1(F). Boxes denote the location of the terminal repeat sequences (ab and ca), reiterated internally in inverted orientation (b'a'c'). *Bam*HI I, *Bam*HI restriction endonuclease fragment containing the gC gene. Line 2 shows expansion of the *Bam*HI fragment with the locations of open reading frames (8). Open reading frame UL44 encodes gC. Line 3 shows expansion of the right-end *Hpa* I–*Bam*HI fragment of *Bam*HI I, containing the gC (UL44) open reading frame. Line 4 shows the location of the deletion contained in R6012. A plasmid containing the *Hpa* I–*Bam*HI subfragment of *Bam*HI I (pRB4162) was digested with *Nhe* I and *Eco*RV, followed by filling-in of the *Nhe* I end with T4 polymerase and ligation. The resulting plasmid (pRB4191) was cotransfected with HSV-1(F) DNA and R6012 was selected from the progeny by screening for plaques not reactive with the anti-gC monoclonal antibody HC1. bp, Base pairs.

infection, and immunoperoxidase-stained using a monoclonal antibody (H126) directed against the viral glycoprotein gB. The titers obtained in one experiment for each virus in MDCK and Vero cells are shown in Table 1. In this and other experiments, HSV-1(F) reproducibly formed small plaques in MDCK cells with an efficiency of 10^{-2} to 10^{-3} as compared to that in Vero cells (Table 1). The gC^+ repaired viruses R6016 and R6017 infected and formed plaques in MDCK cells with an efficiency of approximately 1×10^{-3} as compared to Vero cells (data not shown). Other recombinant viruses (e.g., R3631 and R7041; refs. 3 and 14), lacking the genes encoding infected cell protein 47, the US11 open reading frame, and the viral protein kinase, were also able to infect and form plaques in MDCK cells (data not shown). In five experiments, R6012 gave titers in the MDCK cells 10^8 times lower than in Vero cells (Table 1). In a single experiment, a few plaques were detected in MDCK cells after exposure of the apical surface to an extremely high multiplicity of infection by R6012 [100 plaque-forming units (pfu) per cell]; these were most likely due to the presence of a few nonpolarized cells in the monolayer.

MDCK Cells Apically Exposed to R6012 Virus Do Not Produce Infected Cell Proteins. MDCK cells grown on plastic surfaces were exposed to HSV-1(F) or R6012 at 50 pfu per cell, labeled with [^{35}S]methionine from 19 to 20 hr, and harvested 20 hr after infection. The labeled proteins are shown in Fig. 2. Cells infected with wild-type virus produced a typical pattern of late γ proteins. In cells exposed to R6012, no labeled viral proteins could be detected and no shut-off of host protein synthesis occurred. These proteins after transfer to nitrocellulose, binding to an antibody directed against the α4 protein (H640), and immunoperoxidase staining are shown in Fig. 2 *Upper*. The protein detected, one of the earliest made during infection, is stable and can be detected in cells even late after infection (15). The α4 protein was synthesized in MDCK cells infected with wild-type virus, but not in cells infected with R6012, indicating that the block in gC^- virus replication was prior to the initiation of viral gene expression.

The HSV-1 Glycoprotein gC Is Required for Attachment of Virus to the Apical Surface of MDCK Cells. In one experiment (Fig. 3) confluent cultures of Vero or MDCK cells grown on plastic dishes were exposed to HSV-1(F) or to R6012. At 30-min intervals, portions of the inoculum were removed and titered on Vero cells to determine the amount of virus remaining unattached to cells. On Vero cells (Fig. 3 *Lower*), the removal of R6012 virus from the inoculum by attachment to cells was almost as efficient as that of HSV-1(F). On MDCK cells (Fig. 3 *Upper*), 50% of wild-type virus was removed from the inoculum by 120 min after infection, whereas no significant decrease in R6012 was detected. The

Table 1. Efficiency of plating on Vero and MDCK cells

Virus	Plating efficiency		
	MDCK	Vero	Vero/MDCK
HSV-1(F)	3×10^5	1×10^9	3×10^3
R6012	$<10^2$	1×10^{10}	$>1 \times 10^8$

Ratio of Vero to MDCK titers is shown.

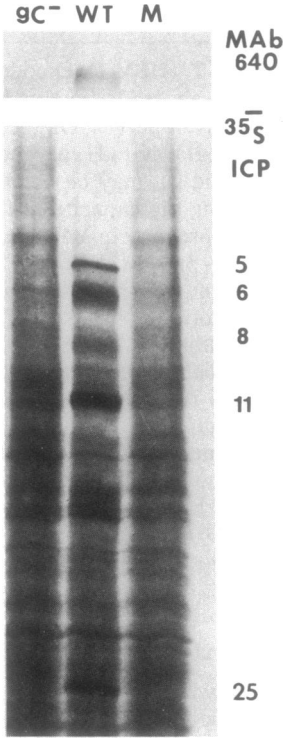

FIG. 2. Electrophoretically separated proteins from MDCK cells mock-infected or infected with HSV-1(F) or with R6012. MDCK cells on plastic dishes were exposed to HSV-1(F) or R6012 at 50 pfu per cell. The cultures were labeled with [^{35}S]methionine (800 Ci/mmol; 100 μCi/ml; 1 Ci = 37 GBq) from 19 to 20 hr after infection. The cells were then harvested, lysed in denaturing buffer, electrophoretically separated in denaturing gels, transferred to nitrocellulose, and autoradiographed on x-ray film (*Lower*) or immunologically detected by incubating with the anti-ICP4 monoclonal antibody H640 and with goat anti-mouse IgG linked to peroxidase and staining with 4-chloro-1-naphthol (*Upper*). MAb, monoclonal antibody; ICP, infected cell polypeptide; gC^-, R6012; WT, HSV-1 (F); M, uninfected cells.

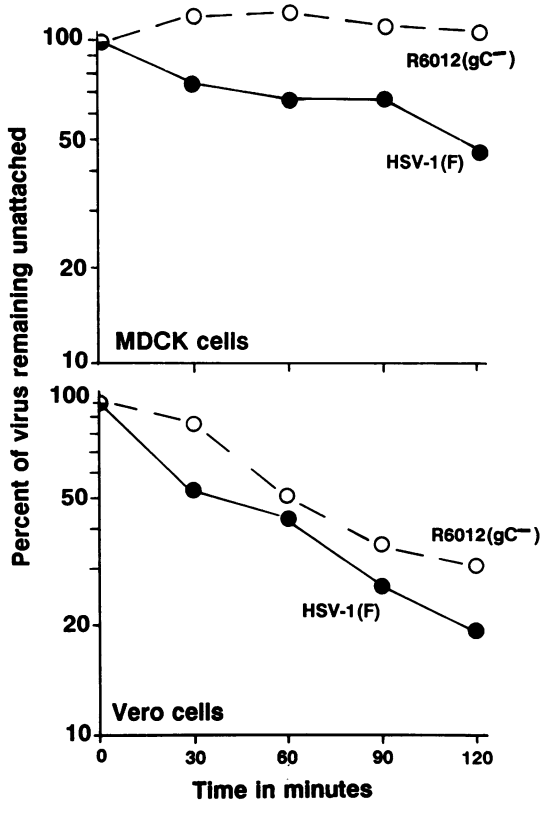

FIG. 3. Attachment of HSV-1(F) and R6012 to Vero or MDCK cells. Virus inoculum (3 ml containing ≈6000 pfu/ml) was added to confluent monolayers of MDCK or Vero cells in 10-cm plastic plates, and then 100 μl was removed from each plate 30, 60, 90, and 120 min after infection and titered on Vero cells. All adsorption assays were done in duplicate. Percent virus remaining unattached was calculated as a percentage of the titer of 100 μl of the initial inoculum. (*Upper*) MDCK cells. (*Lower*) Vero cells.

results obtained in other experiments are shown in Table 2. In these experiments only ≈10% of the R6012 virus appeared

Table 2. Adsorption to Vero and MDCK cells

	% virus unadsorbed after 120 min			
	Vero cells		MDCK cells	
Exp.	HSV-1(F)	R6012	HSV-1(F)	R6012
1*	19	31	46	110
2	2	15	42	90
3	6	14	51	86

*Experiment shown in Fig. 3.

to be removed from the inoculum after 120 min of adsorption. Although we cannot exclude specific attachment to a small number of receptors on apical surfaces of MDCK cells, the decrease in virus in the inoculum could also be due to nonspecific attachment to plastic surfaces, dislodged cells, or inactivation.

gC⁻ Virus Does Infect MDCK Cells from the Basal Surface. MDCK cell monolayers were grown on collagen-coated microporous membrane filters with pore sizes of 0.4 or 3.0 μm. Preliminary experiments indicated that, although the virus was much better able to diffuse through the 3.0-μm pores and infect a larger number of cells, the 0.4-μm pore-size filters allowed for much better cell morphology, easier visualization of plaques, and a lower likelihood of gaps in the monolayer that could lead to leakage of virus between the apical and basal compartments of the filter units. Because of this, quantitative and high multiplicity basal infections were done on cells grown on the 3.0-μm units, and apical and qualitative basal infections were done on cells grown on 0.4-μm units.

Examples of MDCK cell cultures infected by this procedure are shown in Fig. 4. Apical exposure of MDCK cells to ≈100 pfu of HSV-1(F) per cell resulted in the infection of 30–50% of the cells by 48 hr, as detected by immunoperoxidase staining of fixed cell monolayers (Fig. 4*A*). Cells exposed under identical conditions to R6012 failed to show any evidence of infection (Fig. 4*B*). Exposure of the basal surface of MDCK cells to 100 pfu of R6012 per cell yielded the same number of plaques as that obtained with HSV-1(F) under identical conditions. Individual HSV-1(F) and R6012

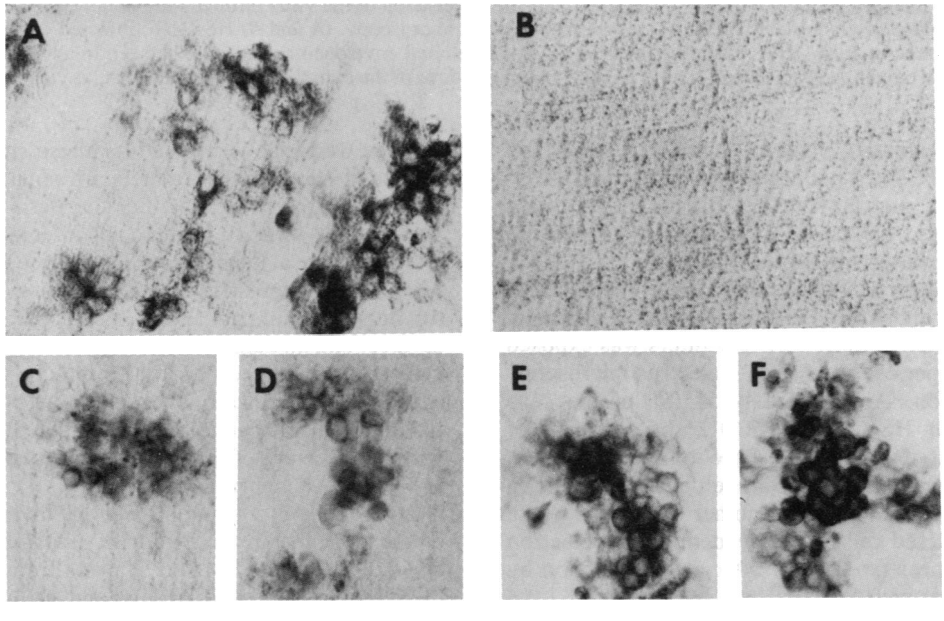

FIG. 4. MDCK cells infected with HSV-1(F) or R6012. Confluent monolayers of MDCK cells grown on collagen-coated Transwell filters (Costar) were infected with HSV-1(F) (*A*, *C*, and *D*) or R6012 (*B*, *E*, and *F*) at 100 pfu per cell. At 48 hr after infection, the filters containing the cells were fixed in methanol and immunoperoxidase-stained using antibody to the viral gB (monoclonal antibody H126). (*A* and *B*) Cells grown on 0.4-μm pore-size filters and exposed to virus from the apical side. (*C–F*) Cells grown on 0.4-μm pore-size filters and exposed to virus from the basal side.

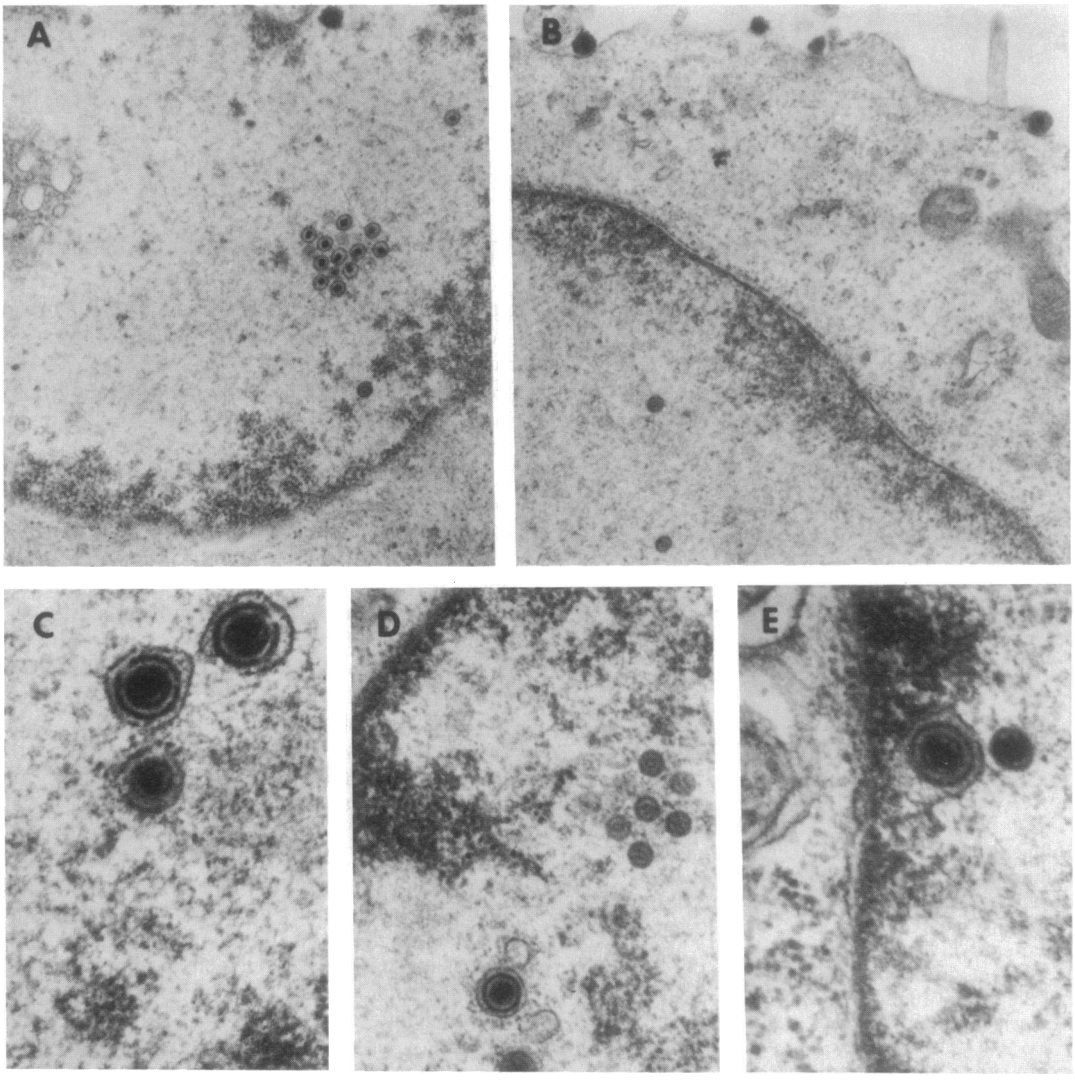

FIG. 5. Electron micrographs of MDCK cells infected with HSV-1(F) or R6012. Cells were grown on 3.0-μm pore-size collagen-coated filters and exposed to virus (1000 pfu per cell) from the basal side to ensure penetration of the virus through the pores. Twenty hours after infection, filters and cells were fixed in glutaraldehyde and processed for electron microscopy. (*A* and *B*) HSV-1(F)-infected cells. Note the enveloped capsids at the cell surface in *B*. (*C–E*) R6012-infected cells. Unenveloped and enveloped capsids can be seen in all three panels. The double membranes seen on the enveloped capsids in *D* and *E* are due to invaginations of the cytoplasm into the nucleus, as can be seen from a different angle in *C*.

immunoperoxidase-stained plaques in MDCK cells are shown in Fig. 5 *C–F*. We have not seen a significant difference between R6012 and HSV-1(F) with respect to plaque number, morphology, or size in MDCK cells exposed to these viruses at the basal surface.

Electron micrographs of MDCK cells basally infected with HSV-1(F) or R6012 are shown in Fig. 5. The basal surface of MDCK cells grown on 3.0-μm pore-size filters was exposed to 1000 pfu of virus per cell and fixed for electron microscopy 24 hr after infection. Approximately 5–10% of the cells infected with either HSV-1(F) or R6012 showed signs of infection as indicated by the presence of nucleocapsids. Enveloped cytoplasmic virions could be detected readily in cells infected with either virus. In another experiment (data not shown), no infected cells could be identified by electron microscopy in cultures fixed 24 hr after apical infection by 100 pfu of R6012 per cell.

DISCUSSION

The significance of the studies presented in this report stems from the conclusive evidence that HSV-1(F), a limited-passage wild-type virus used for these studies, infected and produced viral gene products in polarized MDCK cells exposed to the virus at either the basal or apical surface, whereas gC^- virus attached to and infected MDCK cells only after exposure at the basal surface. These results indicate that MDCK cells express two viral receptors (Fig. 6). One receptor interacts with gC and is located at least at the apical surface, and an additional receptor is found only at the basal cell surface. The receptor for gC may be compartmentalized to the apical surface or be present on both surfaces in the polarized cells, whereas the second receptor, which interacts with one or more of the other glycoproteins, must be sorted to the basal membrane.

An indication of the domain of gC involved in attachment emerged from a reexamination of data published many years ago (19, 20). HSV-1(MP) contains a frame-shift mutation in the gC gene at codon 96 (16) and at least one additional mutation in the open reading frame U_L53 that causes the infected cells to fuse (8, 17, 18). Exposure of MDCK cells to HSV-1(MP) at high multiplicities of infection led to the production of viral structural proteins and DNA but infectious progeny was not made (19, 20). Blind serial passage of

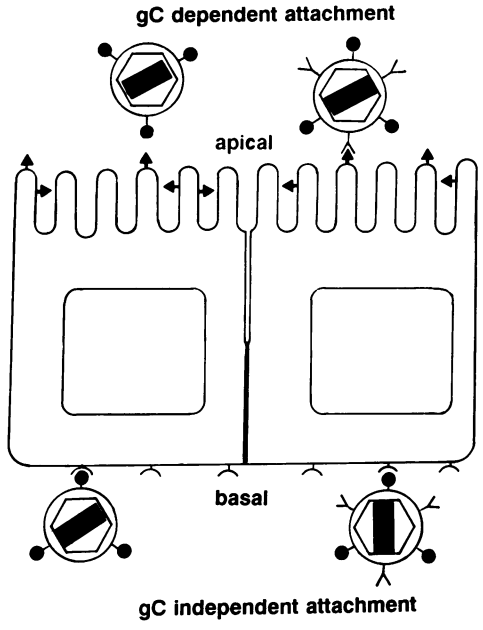

FIG. 6. Asymmetric distribution of HSV receptors on MDCK cells. gC-dependent attachment is represented by gC on the virion (forks) binding to a gC receptor (solid triangle) present on at least the apical cell surface. gC-independent attachment is represented by another viral glycoprotein (solid circles) binding to a different receptor (semicircles) present only on the basal surface of the cells.

the virus in MDCK cells led to the isolation of mutants [HSV-1(MP)dk+] that were able to replicate in those cells (19). Subsequent studies revealed that HSV-1(MP) infection of MDCK cells aborted because envelopment of the virus did not ensue (21). We have found that HSV-1(MP) virus attached to MDCK cells with the same efficiency as the wild-type virus and that the HSV-1(MP)dk+ mutants had reverted to a gC^+ phenotype (data not shown). The inability of HSV-1(MP) to produce infectious progeny in MDCK cells appears to be due to additional defects manifested later in the replicative cycle. The implication of these studies, that the product of the frame-shift mutation containing authentic gC amino acids is sufficient for attachment, requires further study.

In recent years several laboratories have published evidence that gC may play a determinant role in the initial steps of entry of HSV into cells (22–24). Recovery of infectious virus in the extracellular fluid after exposure to the apical surface of MDCK cells to the gC^- mutant constitutes definitive evidence that a receptor for this virus was absent from the apical surface of those cells.

The observations that receptors are sorted differently on polarized cell surfaces and that the virion contains different glycoproteins to recognize the different receptors indicate unambiguously that there is more than one receptor for attachment of HSV-1 to cells and that a single virus utilizes multiple viral glycoprotein–host surface receptor interactions for entering cells. It has been reported (25) that HSV-1 specifically attaches to the receptor of the fibroblast growth factor. At this time we do not know which, if any, of our receptors corresponds to the fibroblast growth factor receptor. Our finding of more than one cellular receptor–viral glycoprotein interaction does explain the observation that neither fibroblast growth factor (25) nor other competitive inhibitors of HSV attachment (12, 22, 26, 27) have been able to completely ablate the attachment of HSV-1 to cells.

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