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Role of Nectin-1 and Herpesvirus Entry Mediator as Cellular Receptors for Herpes Simplex Virus 1 on Primary Murine Dermal Fibroblasts

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ABSTRACT

The cellular proteins nectin-1 and herpesvirus entry mediator (HVEM) can both mediate the entry of herpes simplex virus 1 (HSV-1). We have recently shown how these receptors contribute to infection of skin by investigating HSV-1 entry into murine epidermis. *Ex vivo* infection studies reveal nectin-1 as the primary receptor in epidermis, whereas HVEM has a more limited role. Although the epidermis represents the outermost layer of skin, the contribution of nectin-1 and HVEM in the underlying dermis is still open. Here, we analyzed the role of each receptor during HSV-1 entry in murine dermal fibroblasts that were deficient in expression of either nectin-1 or HVEM or both receptors. Because infection was not prevented by the absence of either nectin-1 or HVEM, we conclude that they can act as alternative receptors. Although HVEM was found to be highly expressed on fibroblasts, entry was delayed in nectin-1-deficient cells, suggesting that nectin-1 acts as the more efficient receptor. In the absence of both receptors, entry was strongly delayed leading to a much reduced viral spread and virus production. These results suggest an unidentified cellular component that acts as alternate but inefficient receptor for HSV-1 on dermal fibroblasts. Characterization of the cellular entry mechanism suggests that HSV-1 can enter dermal fibroblasts both by direct fusion with the plasma membrane and via endocytic vesicles and that this is not dependent on the presence or absence of nectin-1. Entry was also shown to require dynamin and cholesterol, suggesting comparable entry pathways in keratinocytes and dermal fibroblasts.

IMPORTANCE

Herpes simplex virus (HSV) is a human pathogen which infects its host via mucosal surfaces or abraded skin. To understand how HSV-1 overcomes the protective barrier of mucosa or skin and reaches its receptors in tissue, it is essential to know which receptors contribute to the entry into individual skin cells. Previously, we have explored the contribution of nectin-1 and herpesvirus entry mediator (HVEM) as receptors for HSV-1 entry into murine epidermis, where keratinocytes form the major cell type. Since the underlying dermis consists primarily of fibroblasts, we have now extended our study of HSV-1 entry to dermal fibroblasts isolated from nectin-1- or HVEM-deficient mice or from mice deficient in both receptors. Our results demonstrate a role for both nectin-1 and HVEM as receptors and suggest a further receptor which appears much less efficient.

To initiate infection, herpes simplex virus 1 (HSV-1) enters its human host via mucosal surfaces or abraded skin. HSV-1 entry into individual cells involves the interaction of several viral glycoproteins with various cell surface receptors (1, 2). The first step during entry is the attachment of virions to glycosaminoglycans, which facilitates the interaction with cellular receptors, leading to the fusion of the viral envelope with a cellular membrane. Fusion can either occur with the plasma membrane or with vesicle membranes after virions are internalized via endocytosis (3, 4). Only after binding of the envelope glycoprotein D (gD) to a receptor is fusion with cellular membranes induced (5). The primary gD receptors mediating entry into mouse and human cells are nectin-1 and herpesvirus entry mediator (HVEM) (6–8). The 3-O-sulfated heparan sulfate (3-OS-HS) represents a further gD receptor, which may also contribute to HSV-1 entry into various cell types (9, 10).

How each of these receptors contributes to the entry process of HSV-1 into natural target sites such as skin or mucosa is not well understood. Since the absence of both nectin-1 and HVEM prevents HSV pathogenesis in the mouse model, nectin-1 and HVEM are reported to be the dominant functional gD receptors in the murine host (11–13). Using nectin-1- or HVEM-deficient mice, we recently investigated HSV-1 entry into murine epidermis. Our *ex vivo* infection studies identified nectin-1 as the major receptor in the epidermis, whereas HVEM has a more limited role (14). Since the epidermis represents only the outermost layer of skin

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and mucosa, we address here the contribution of nectin-1 and HVEM as receptors in the underlying dermis. Fibroblasts are the major resident cell type of the dermis, which is connected to the epidermis through the basement membrane, a specialized layer of extracellular matrix that anchors the keratinocytes (15).

Nectin-1 is a Ca^{2+}-independent immunoglobulin-like cell-cell adhesion molecule involved in the formation of adherens junctions in epithelial cells and fibroblasts (16). In fibroblasts, nectin-1 is detectable at cell-cell adhesion sites and perhaps also diffusely distributed on the free surface of the plasma membrane of migrating cells (17). As a member of the tumor necrosis factor receptor superfamily, HVEM can activate either proinflammatory or inhibitory signaling pathways (18). This receptor is expressed mainly by T lymphocytes but is also present on B cells, natural killer cells, dendritic cells, and fibroblasts (19–21). HVEM is only expressed at low levels on human dermal fibroblasts (22).

Using nectin-1- or HVEM-deficient murine dermal fibroblasts, we investigated the role of nectin-1 and HVEM as receptors for HSV-1 and characterized the uptake mechanism. Our results demonstrate that HSV-1 can enter into nectin-1-deficient fibroblasts, indicating that HVEM is able to replace nectin-1 as a receptor. However, infection was delayed in the absence of nectin-1, and virus growth and spread was less efficient. In the absence of both nectin-1 and HVEM, infection was still observed, although it was severely delayed, suggesting the presence of a further receptor. Irrespective of whether nectin-1 was present, HSV-1 entry into dermal fibroblasts required dynamin and cholesterol, and uptake seemed to involve both direct fusion with plasma membrane and endocytic vesicles.

MATERIALS AND METHODS

Mice, preparation of murine skin and isolation of murine fibroblasts. Breeding of $Pvrl^{-/-}$ mice lacking nectin-1 was recently described (14, 23). Murine skin samples were taken from the backs of wild-type (wt) (C57BL/6), $Pvrl^{-/-}$ (23), or HVEM knockout (KO) newborn mice (24), referred to here as wt mice, nectin-1-deficient mice, or HVEM-deficient mice, respectively. Murine skin was also taken from tails of wt (C57BL/6) or $Tnfrsf14^{-/-}/Pvrl1^{-/-}$ adult (10-month-old) mice (11), referred to here as wt or double-KO mice, respectively. The dermis of newborn and adult skin was removed from the epidermis by dispase II treatment, as described previously (14). To isolate dermal fibroblasts, the dermis was shaken gently for 60 min (newborn dermis) or 3 h (adult dermis) at 37°C in serum-free Dulbecco's modified Eagle medium (DMEM)-high glucose-GlutaMAX (Life Technologies) containing 400 U of collagenase I (Worthington)/ml. Primary fibroblasts were cultured in DMEM-high glucose-GlutaMAX (Life Technologies) containing 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Experiments were typically performed in primary fibroblasts at passages 2 to 4 after isolation.

Virus. Infection studies were performed with purified preparations of HSV-1 wt strain 17, as described previously (25). In brief, virus inoculum was added to the cells at 37°C defining time point zero. For electron microscopy (EM) studies, primary fibroblasts were incubated with HSV-1 at 800 PFU/cell for 1 h at 4°C, followed by incubation at 37°C for 10 or 30 min to allow uptake.

Virus titers of cell released virus were determined by plaque assays on Vero-B4 cells. To determine virus spreading, confluent monolayers of murine primary fibroblasts were infected with HSV-1 at 0.1 PFU/cell. At 30, 75, 180, and 360 min postinfection (p.i.) medium was replaced by 0.5% human pooled serum and incubation continued until 24 h p.i., followed by counting the plaques. Virus production was determined at 24, 30, 36, 48, and 60 h p.i.

Ethics statement. The preparation of dermal cells from sacrificed animals was carried out in strict accordance with the recommendations of the Guide of Landesamt für Natur, Umwelt, and Verbraucherschutz, Northrhine-Westphalia (Germany). The study was approved by LANUV NRW (8.84-02.05.20.13.018).

Inhibitor studies. The dynamin inhibitor dynasore (Tocris) was dissolved in dimethyl sulfoxide (DMSO), and methyl-β-cyclodextrin (MβCD; Sigma) was dissolved in water. The tested concentrations of the drugs had no effect on cell viability as shown by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays (data not shown). Cells were treated with the appropriate drugs for 30 min at 37°C before infection. Dynasore was present throughout infection, but MβCD was removed prior to infection by washing the cells with medium three times.

RNA preparation and reverse transcription-PCR (RT-PCR). TRIzol reagent (Life Technologies) was used to extract RNA from murine primary dermal fibroblasts and from the murine melanoma cell line B16-F1 (26), obtained from Anika Steffen, Braunschweig. cDNAs were synthesized from total RNA by using the SuperScript II reverse transcriptase (Life Technologies), and PCR was performed with native *Taq* DNA polymerase (Life Technologies) and the following primer pairs: nectin-1 primers (forward, 5′-ACTGGTTTCTGGAGCGCGAGG-3′; reverse, 5′-CTCGTAGGGAGGCAGCACGGA-3′), nectin-2 primers (forward, 5′-AGCTGGGCCGAACGAACTGATC-3′; reverse, 5′-AGCCACA ACTGTGCCATCCAGG-3′), nectin-3 primers (forward, 5′-TATGCAAA GCCGTTACATTCCC-3′; reverse, 5′-TGGCTGACAATCGTTGCTGTT T-3′), nectin-4 primers (forward, 5′-GGCAGCTTTCAGGCACGGAT-3′; reverse, 5′-GGCACCAGATGGAACTCTGAAG-3′), HVEM primers (forward, 5′-TGAAGCAGGTCTGCAGTGAG-3′; reverse, 5′-GCTGTTG GTCCCACGTCTTA-3′), 3-OST-3A1/3B1 primers (27) (forward, 5′-CA GGCCATCATCATCGG-3′; reverse, 5′-CCGGTCATCTGGTAGAA-3′), and GAPDH primers (forward, 5′-TGATGACATCAAGAAGGTGGTGA AG-3′; reverse, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′).

Immunocytochemistry and antibodies. Murine primary fibroblasts grown on uncoated coverslips were fixed with 2% formaldehyde for 10 min at room temperature, permeabilized with 0.5% NP-40 for 10 min at room temperature, and then stained for 60 min with mouse anti-ICP0 (monoclonal antibody [MAb] 11060) (28) diluted 1:60, followed by incubation with AF488-conjugated anti-mouse IgG (Life Technologies) and DAPI (4′,6′-diamidino-2-phenylindole) for 45 min at room temperature. Staining of F-actin was performed with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) for 15 min at room temperature. Microscopy was performed using a Leica DM IRB/E microscope linked to a Leica TCS-SP/5 confocal unit. Images were assembled using Photoshop (version CS2; Adobe).

Transmission electron microscopy. Infected cells were prepared for electron microscopy as described previously (29). Ultrathin sections of fibroblasts were cut, stained with uranyl acetate and lead citrate, and analyzed in a JEOL 1200 EX II.

Flow cytometric analysis. Murine primary fibroblasts from wt, nectin-1-deficient, or HVEM-deficient mice were mock infected or infected for 1 or 3 h, followed by detachment with 0.05% trypsin–0.02% EDTA. The detached fibroblasts were dispersed by gentle pipetting and filtered one time through a 40-μm-pore-size cell strainer (BD) to remove cell clumps and debris. To stain surface receptors, cells were kept in phosphate-buffered saline–5% FCS and incubated on ice for 45 min with Armenian hamster MAb against murine HVEM (clone HMHV-1B18; BioLegend) diluted 1:200, mouse anti-nectin-1 (CK41) antibody (30) diluted 1:100, or isotype controls: for HVEM, Armenian hamster IgG (eBioscience), and for nectin-1, mouse IgG1 (Life Technologies). This was followed by incubation with the secondary antibodies, anti-Armenian hamster IgG (phycoerythrin [PE]; eBioscience) diluted 1:50 or anti-mouse IgG (Cy5; Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 for 30 min on ice. To visualize ICP0, the cells were fixed with 3.7% formaldehyde for 10 min at room temperature, permeabilized with 0.2% saponin for 15

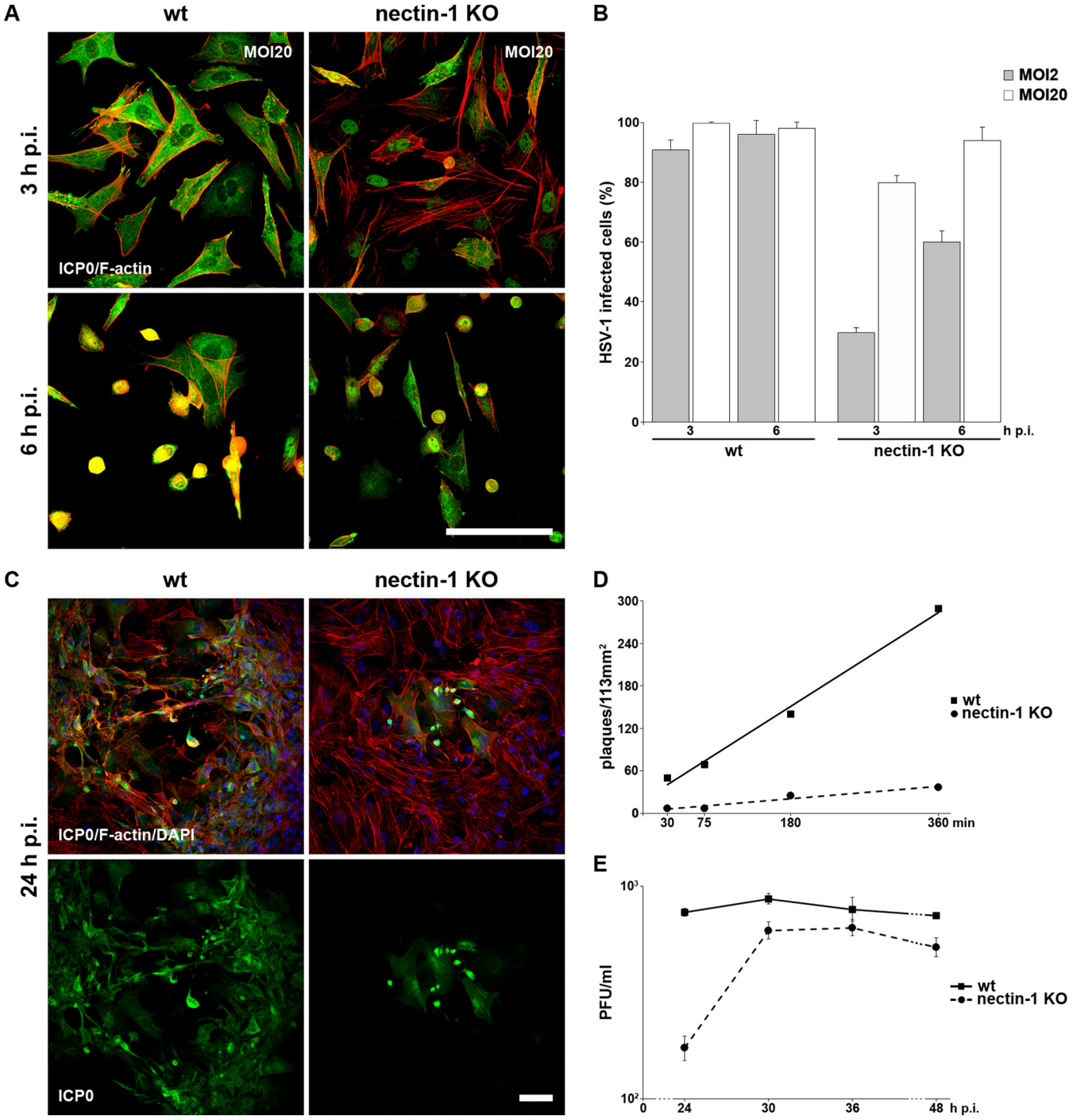


FIG 1 HSV-1 enters murine wt and nectin-1-deficient primary dermal fibroblasts. (A) Primary fibroblasts from wt or nectin-1-deficient (nectin-1 KO) newborn mice were infected. F-actin was visualized by phalloidin (red) and infected cells by staining for ICP0 (green). Confocal projections and merged images show that all wt cells and a majority of nectin-1-deficient cells were infected at 3 h p.i. At 6 h p.i., CPE was mainly visible in wt cells. Bar, 100 μm. (B) Wild-type (wt) or nectin-1-deficient primary fibroblasts were infected, and the number of ICP0-expressing cells was determined at 3 and 6 h p.i. in at least three independent experiments. The results are shown as means ± standard deviations. MOI, multiplicity of infection. (C) To analyze viral spread, wt and nectin-1-deficient fibroblasts were preincubated with HSV-1 at 0.1 PFU/cell for 75 min, at which time the inoculum was replaced by medium containing human anti-HSV-1 serum. After staining with anti-ICP0 (green) and phalloidin (red) and counterstaining of nuclei with DAPI (blue) at 24 h p.i., confocal projections and merged images demonstrate much smaller plaques in nectin-1-deficient than in wt cells. Bar, 100 μm. (D) At 30, 75, 180, and 360 min p.i., the medium was replaced by 0.5% human pooled serum, and incubation continued until 24 h p.i. We show the smaller plaque formation in nectin-1-deficient cells in three independent experiments. The results are means ± the standard deviations. (E) To measure virus production, wt and nectin-1-deficient fibroblasts were infected at 0.1 PFU/cell, and the virus titer was determined at various times postinfection in three independent experiments. The results demonstrate delayed production of HSV-1 in the absence of nectin-1. The results are shown as means ± standard deviations.

min on ice, and then incubated on ice for 35 min with mouse anti-ICP0 (MAb 11060) (28) diluted 1:30 in 0.2% saponin or with the isotype control mouse IgG2b (Life Technologies), followed by incubation on ice for 30 min with anti-mouse IgG (Cy5; Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 in 0.2% saponin. Samples were analyzed by using a FACSCanto II flow cytometer (BD) and FACSDiva (v6.1.3; BD) and FlowJo (v7.6.3; Tree Star) software.

RESULTS

Entry of HSV-1 into nectin-1-deficient primary dermal fibroblasts. To explore the contribution of nectin-1 to HSV-1 entry into dermal fibroblasts, we isolated fibroblasts from newborn skin of either wt or nectin-1-deficient mice and performed infection studies. Successful HSV-1 entry was visualized by staining with an antibody against the viral immediate-early protein ICP0. The cellular localization of ICP0 passes through distinct phases during early infection; ICP0 in nuclear foci indicates an early stage of viral gene expression, which is followed by the relocalization of ICP0 to the cytoplasm (31). After infection for 3 h with 20 PFU/cell, we detected cytoplasmic ICP0 in all fibroblasts from wt mice and by 6 h p.i. cytopathic effects (CPEs) were already visible (Fig. 1A and B). In nectin-1-deficient fibroblasts there was a slight decrease in the numbers of ICP0-expressing cells at 3 h p.i. but by 6 h p.i. the numbers were comparable to wt mice (Fig. 1A and B). The decrease seen in nectin-1-deficient cells was more prominent when infection was performed at 2 PFU/cell, but the numbers of ICP0-expressing cells increased with time, indicating a delay of infection in the absence of nectin-1 (Fig. 1B). The predominantly cytoplasmic localization of ICP0 in wt cells at 3 h p.i. also demonstrates that infection progressed more rapidly than in nectin-1-deficient cells, where ICP0 was largely confined to nuclei. Further evidence of a delay in nectin-1-deficient cells is provided by the reduced onset of cytopathic effects at 6 h p.i. (Fig. 1A). These results demonstrate that the lack of nectin-1 does not prevent entry into dermal fibroblasts but suggest that the infection process is slowed down (Fig. 1B).

To investigate whether the absence of nectin-1 in dermal fibroblasts influences cell-to-cell spread of HSV-1, we analyzed plaque formation. Cells were preincubated with HSV-1 for different times before being overlaid with medium containing HSV-1 antiserum. After further incubation for 24 h, the cells were fixed, and plaques were visualized by staining with anti-ICP0 antibodies. Under these conditions, plaques were notably smaller and their number was significantly reduced in the absence of nectin-1 compared to wt fibroblasts (Fig. 1C and D), indicating that viral spread was greatly reduced. As a consequence of the reduced viral spread, we also observed delayed and reduced virus production (Fig. 1E).

Taken together, HSV-1 infection of nectin-1-deficient dermal fibroblasts was less efficient. Although HSV-1 could still enter cells in the absence of nectin-1, the HSV-1 entry process was delayed, and viral spread was reduced, resulting in less virus production.

Expression of HVEM and nectins on wt and nectin-1-deficient dermal fibroblasts. Since the absence of nectin-1 did not abolish infection of primary dermal fibroblasts, we investigated the presence of HVEM as a possible alternative receptor. Initially, we confirmed the lack of nectin-1 in nectin-1-deficient fibroblasts and demonstrated the presence of nectin-2 and -3, as well as small amounts of nectin-4 in both wt and nectin-1-deficient fibroblasts, by RT-PCR (Fig. 2). It is unlikely that these nectins serve as alternative receptors, since the specificity of murine nectin-2 is restricted to pseudorabies virus (32, 33), and nectin-3 and -4 exhibit no known receptor activity for wt alphaherpesviruses in the murine host. HVEM RNA was readily detected in either the presence or the absence of nectin-1 (Fig. 2).

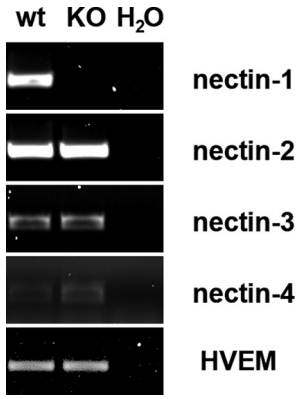
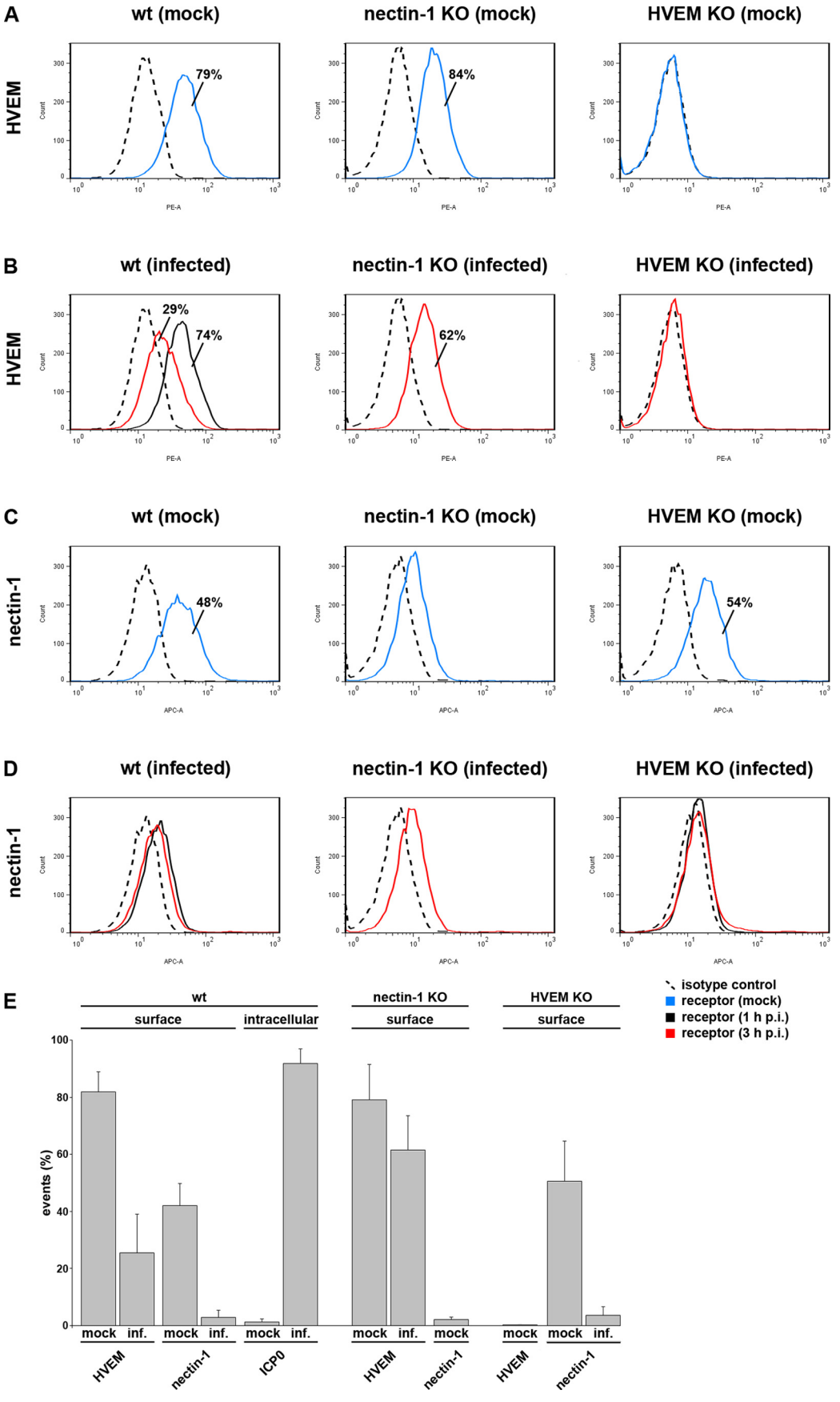


FIG 2 Expression of nectins and HVEM in wt and nectin-1-deficient primary dermal fibroblasts. RNA was isolated from wt or nectin-1-deficient fibroblasts. RT-PCR results demonstrate the expression of nectin-2, -3, -4, and HVEM both in wt and nectin-1-deficient cells, whereas nectin-1 was only detected in wt cells. The water controls (H_2O) contained no cDNA.

If HVEM serves as receptor for HSV-1, it should be expressed on the surfaces of dermal fibroblasts. Flow cytometric analysis demonstrated that HVEM was present on ca. 79 and 84% of wt and nectin-1-deficient cells, respectively, indicating its presence on the surfaces of most dermal fibroblasts (Fig. 3A and E). As a control, the anti-HVEM MAb did not recognize any epitopes on HVEM-deficient fibroblasts (Fig. 3A). When we investigated cell surface expression of nectin-1, it was detected on 48 and 54% of wt and HVEM-deficient fibroblasts, respectively (Fig. 3C and E). The specificity of the anti-nectin-1 antibody CK41 was shown in nectin-1-deficient fibroblasts (Fig. 3C).

Taken together, the comparison of HVEM and nectin-1 on the cell surface demonstrated fewer wt fibroblasts with detectable nectin-1 expression than with detectable HVEM (Fig. 3E). To correlate surface expression of nectin-1 with infection efficiency, we infected HVEM-deficient fibroblasts with HSV-1 at 2 or 20 PFU/cell. The infection studies revealed the same high number of ICP0-expressing cells in HVEM-deficient as in wt fibroblasts without the delay in expression of ICP0 that was seen in nectin-1-deficient cells (Fig. 1B and Fig. 4). The very efficient entry in the absence of HVEM might be explained by the presence of nectin-1 on most fibroblasts, although the levels were too low to be detected on all cells by flow cytometry. These results, in turn, suggest that nectin-1 acts as a primary receptor on wt dermal fibroblasts with HVEM acting as a less efficient receptor.

During HSV-1 entry, binding of viral gD to both nectin-1 and HVEM induces receptor downregulation from the cell surface (34–36). If both receptors are used in dermal fibroblasts, the surface expression of nectin-1 and HVEM should be reduced upon infection. Flow cytometric analysis demonstrated that the number of HVEM-expressing cells was reduced to ca. 29% of wt fibroblasts by 3 h p.i. (Fig. 3B and E). Surprisingly, the downregulation of HVEM from the surface of nectin-1-deficient fibroblasts was less pronounced (Fig. 3B and E). These results suggest that HVEM can serve as receptor when nectin-1 is present, as well as in its absence. After infection, nectin-1 was rapidly lost from the cell surface of wt


A
wt (mock)
nectin-1 KO (mock)
HVEM KO (mock)
HVEM
79%
84%
Count
PE-A
B
wt (infected)
nectin-1 KO (infected)
HVEM KO (infected)
HVEM
29%
74%
62%
Count
PE-A
C
wt (mock)
nectin-1 KO (mock)
HVEM KO (mock)
nectin-1
48%
54%
Count
APC-A
D
wt (infected)
nectin-1 KO (infected)
HVEM KO (infected)
nectin-1
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APC-A
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surface
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receptor (mock)
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100
80
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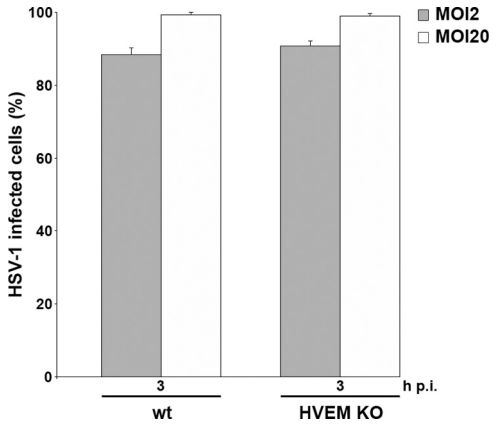

FIG 4 Efficiency of HSV-1 infection in primary fibroblasts. The efficiency of infection was analyzed in wt and HVEM-deficient fibroblasts. At 3 h p.i., the number of ICP0-expressing cells was determined in three independent experiments demonstrating a nearly identical number of infected cells in the presence or absence of HVEM. The results are shown as means ± standard deviations. MOI, multiplicity of infection.

fibroblasts, becoming undetectable by 1 h p.i. (Fig. 3D). In contrast, HVEM was detected on the surface of wt cells for longer, with no obvious loss evident by 1 h p.i. (Fig. 3B).

In summary, nectin-1 disappeared rapidly from the cell surface, whereas HVEM downregulation was slower and perhaps less efficient (Fig. 3E). The internalization of each of the proteins from the cell surface suggests that both nectin-1 and HVEM act as receptors for HSV-1, although with differing efficiencies. Since we observed a slower infection with reduced virus production in the absence of nectin-1 (Fig. 1B and E), we suggest that nectin-1 accelerates HSV-1 entry but that HVEM can replace nectin-1 as a receptor in nectin-1-deficient dermal fibroblasts.

Entry of HSV-1 into nectin-1- and HVEM-deficient dermal fibroblasts. To investigate whether HVEM is the only molecule that acts as receptor in the absence of nectin-1, we infected primary dermal fibroblasts deficient in both nectin-1 and HVEM. After infection for 3 h with 20 PFU/cell, ICP0 expression was detected in 95% of the wt fibroblasts but in only 4% of the double-KO cells, with an increase to 25% of infected cells by 6 h p.i. (Fig. 5A). At 2 PFU/cell, almost no ICP0-expressing cells were detected in the double KO by 6 h p.i. compared to 80% expressing wt cells (Fig. 5A). Interestingly, we observed plaque formation in the double-KO cells at 18 h p.i. (Fig. 5B). This observation implies cell-to-cell spread from single infected cells, which in turn suggests that although cells were exposed to 20 PFU/cell, not all were susceptible to initial infection by HSV-1. In contrast, cytopathic effects and detachment of cells had already started in the corresponding wt cells at 6 h p.i. (Fig. 5B). As expected, we observed severely reduced virus production in double-KO cells compared to the corresponding wt cells (Fig. 5C). Taken together, our results indicate the presence of a receptor that is dramatically less efficient than nectin-1 and HVEM. Since 3-OS-HS has been described to act as receptor for HSV-1 in human corneal fibroblasts (27), we analyzed the presence of the 3-OS-HS generating enzyme 3-O sulfotransferase 3 (3-OST-3) by RT-PCR. In fibroblasts isolated from newborn or adult skin, the 3-OST-3 isoform was transcribed, suggesting the presence of 3-OS-HS on these cells (Fig. 5D). No 3-OST-3 signal was detectable in the control mouse melanoma cell line B16-F1 (Fig. 5D), which is resistant to HSV-1 entry (data not shown). Thus, 3-OS-HS might represent a potential receptor on murine dermal fibroblasts.

Uptake of HSV-1 into primary dermal fibroblasts. To characterize the uptake mechanism into primary dermal fibroblasts in the absence of nectin-1, we performed EM studies of infected nectin-1-deficient fibroblasts and compared the mode of internalization to that in wt cells. In wt fibroblasts, where nectin-1 is expected to act as the most efficient receptor, we found virus particles attached to the cell surface, and free capsids in the cytoplasm, frequently in close proximity to the plasma membrane (Fig. 6Aa and b). In addition, virus particles were sometimes visible in vesicles (Fig. 6Ab). The analysis of nectin-1-deficient fibroblasts revealed a similar distribution of free capsids in the cytoplasm and of virus particles in vesicles to that in wt cells (Fig. 6Aa and d). Since we conclude that nectin-1 serves as primary receptor in wt fibroblasts and HVEM as a further efficient receptor in nectin-1-deficient fibroblasts, we infer that nectin-1 and HVEM promote both direct fusion with the plasma membrane and fusion after uptake via vesicles.

Previously, we have shown that uptake into keratinocytes, the major cell type of the epidermis, depends on the multidomain GTPase, dynamin (37). To further characterize the internalization pathway in primary fibroblasts, we investigated the role of dynamin. When fibroblasts were treated prior to infection with dynasore, a small-molecule inhibitor of the dynamin GTPase activity (38), a concentration-dependent decrease in the number of ICP0-expressing cells was observed both in the presence or absence of nectin-1 (Fig. 6B). Thus, we conclude that HSV-1 uptake into wt and nectin-1-deficient fibroblasts is dynamin dependent.

We have also previously shown that cholesterol is required for HSV-1 uptake into human primary keratinocytes, highlighting the role of cholesterol-rich lipid rafts (37). To investigate the role of cholesterol in primary dermal fibroblasts, we treated them with MβCD, which depletes cholesterol from the plasma membrane. When cells were pretreated with increasing concentrations of MβCD prior to infection, we observed a considerable decrease in the number of ICP0-expressing cells in both wt and nectin-1-deficient cells (Fig. 6C). These results suggest that cholesterol plays

FIG 3 Characterization of nectin-1 and HVEM expression on the surface of wt, nectin-1- deficient, or HVEM-deficient primary dermal fibroblasts. (A and B) Flow cytometric analyses demonstrate that HVEM is detected on 79% of mock-infected wt fibroblasts and on 84% of mock-infected nectin-1-deficient cells. After infection at 100 PFU/cell for 3 h, the surface expression of HVEM is reduced. Although 74% of the wt cells show HVEM at 1 h p.i., expression was reduced to 29% of the cells at 3 h p.i. HVEM is still detected on 62% of the nectin-1-deficient cells at 3 h p.i. As a control, no HVEM was detected on HVEM-deficient cells. (C and D) Flow cytometric analyses indicate that nectin-1 is present on 48 and 54% of mock-infected wt and HVEM-deficient cells, respectively. After infection at 100 PFU/cell, nectin-1 disappears from the surfaces of wt and HVEM-deficient cells already at 1 h p.i. As a control for the specificity of the antibody, nectin-1-deficient cells were stained. Representative flow cytometry histograms are shown. (E) Summary of flow cytometric analyses from at least three independent experiments, indicating the surface expression of HVEM or nectin-1 and intracellular expression of ICP0 in mock-infected and HSV-1-infected wt fibroblasts. HVEM and nectin-1 expression in mock-infected or infected nectin-1- or HVEM-deficient fibroblasts is shown for comparison. The results are shown as means ± standard deviations.

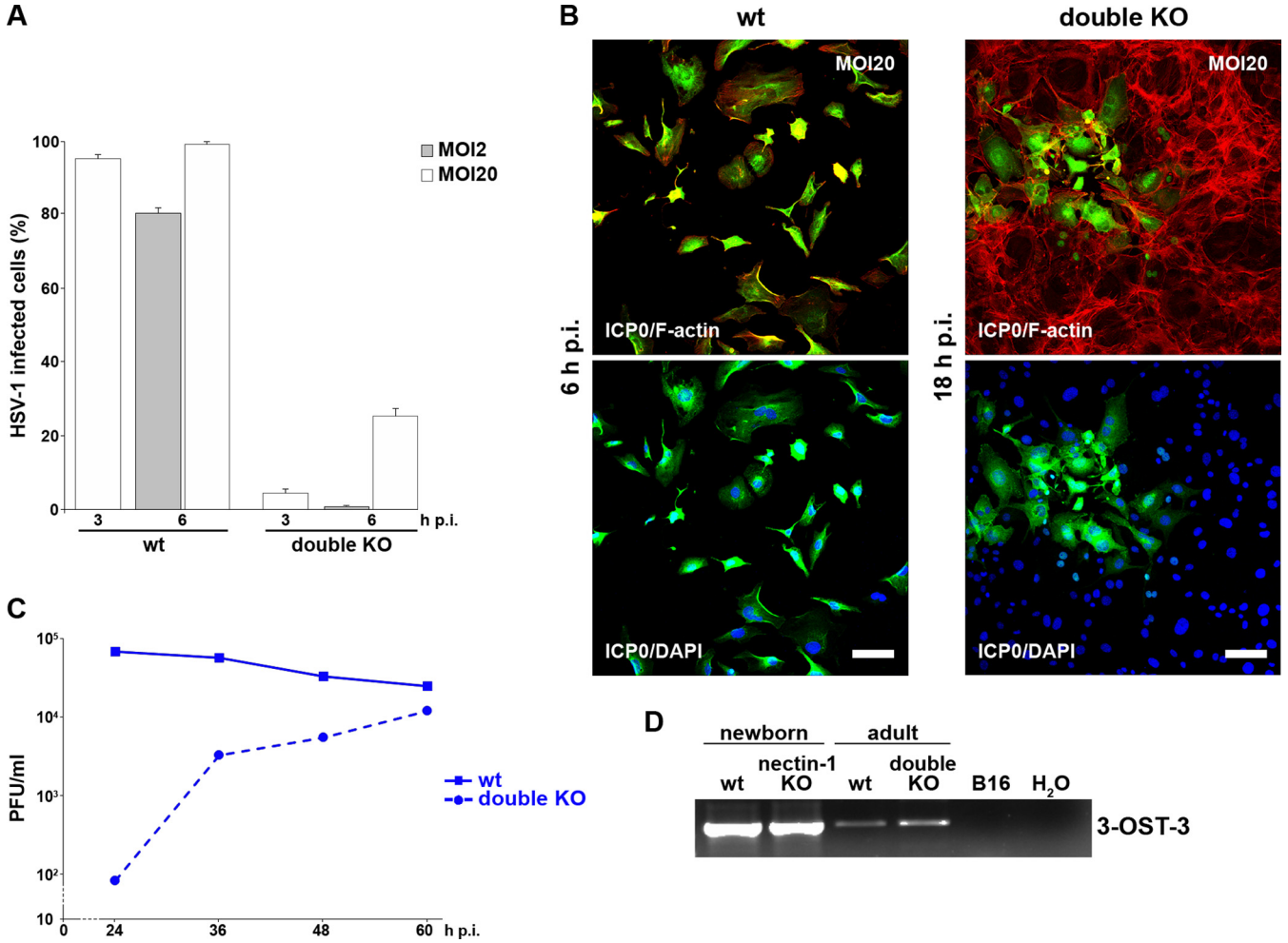

FIG 5 HSV-1 can enter primary dermal fibroblasts deficient in nectin-1 and HVEM at low efficiency. (A) Primary fibroblasts from wt or nectin-1/HVEM-deficient (double-KO) adult tail skin were infected and the number of ICP0-expressing cells was determined at 3 and 6 h p.i. in at least three independent experiments. The results are shown as means ± standard deviations. MOI, multiplicity of infection. (B) Infected wt or double-KO fibroblasts were stained with anti-ICP0 (green), DAPI (blue) and phalloidin (red). Confocal projections and merged images show that plaques are formed in double-KO cells at 18 h p.i. All wt cells are infected at 6 h p.i. and show a CPE. Bar, 100 μm. (C) Virus production was measured after infection of wt and double-KO cells with 0.1 PFU/cell. The virus titer was determined at various times postinfection in two independent experiments. (D) RNA was isolated from wt or double-KO fibroblasts derived from newborn back skin or adult tail skin, or from B16-F1 cells. The results of RT-PCR demonstrate expression of 3-OST-3 in fibroblasts but not in B16-F1 cells. The water control (H_2O) contained no cDNA.

a role in dermal fibroblasts irrespective of whether or not nectin-1 is present.

The absence of nectin-1 led to a slower HSV-1 entry process, which is probably due to delayed viral uptake via HVEM or an alternate receptor. However, the mode of internalization and the dependence on dynamin and cholesterol did not change in the absence or presence of nectin-1, suggesting that entry into nectin-1-deficient dermal fibroblasts uses comparable modes of uptake.

DISCUSSION

During primary infection in skin or mucosa, HSV-1 replicates in epidermal keratinocytes, leading to virally mediated cell death and associated inflammation. Virus-containing fluid accumulates between the epidermal and dermal layers, while an intense inflammatory response takes place in the dermis. This process exposes fibroblasts in the dermis to infection with HSV-1. HSV-1 can also gain direct access to dermal fibroblasts by penetrating abraded skin or via microlesions. To investigate infection of the dermis, we analyzed the contribution of individual receptors for HSV-1 on dermal fibroblasts in order to complement our recent studies in murine epidermis (14).

After the infection of primary dermal fibroblasts, we observed that entry was slower in the absence of nectin-1 than when it was present, although the number of infected cells was not reduced. Since all dermal fibroblasts can support infection in the absence of nectin-1, we analyzed the expression of HVEM as an alternative receptor. HVEM was detected on nearly all dermal fibroblasts while nectin-1 was less easily detected. However, as judged from the ICP0 expression patterns, the entry steps were slower in nectin-1-deficient fibroblasts than in wt fibroblasts. When we investigated viral uptake by EM, we found that the mode of uptake did not change in the absence of nectin-1 with both free cytoplasmic capsids and particles in vesicles. In addition, efficient entry was found to depend on cholesterol and dynamin, the effects of which

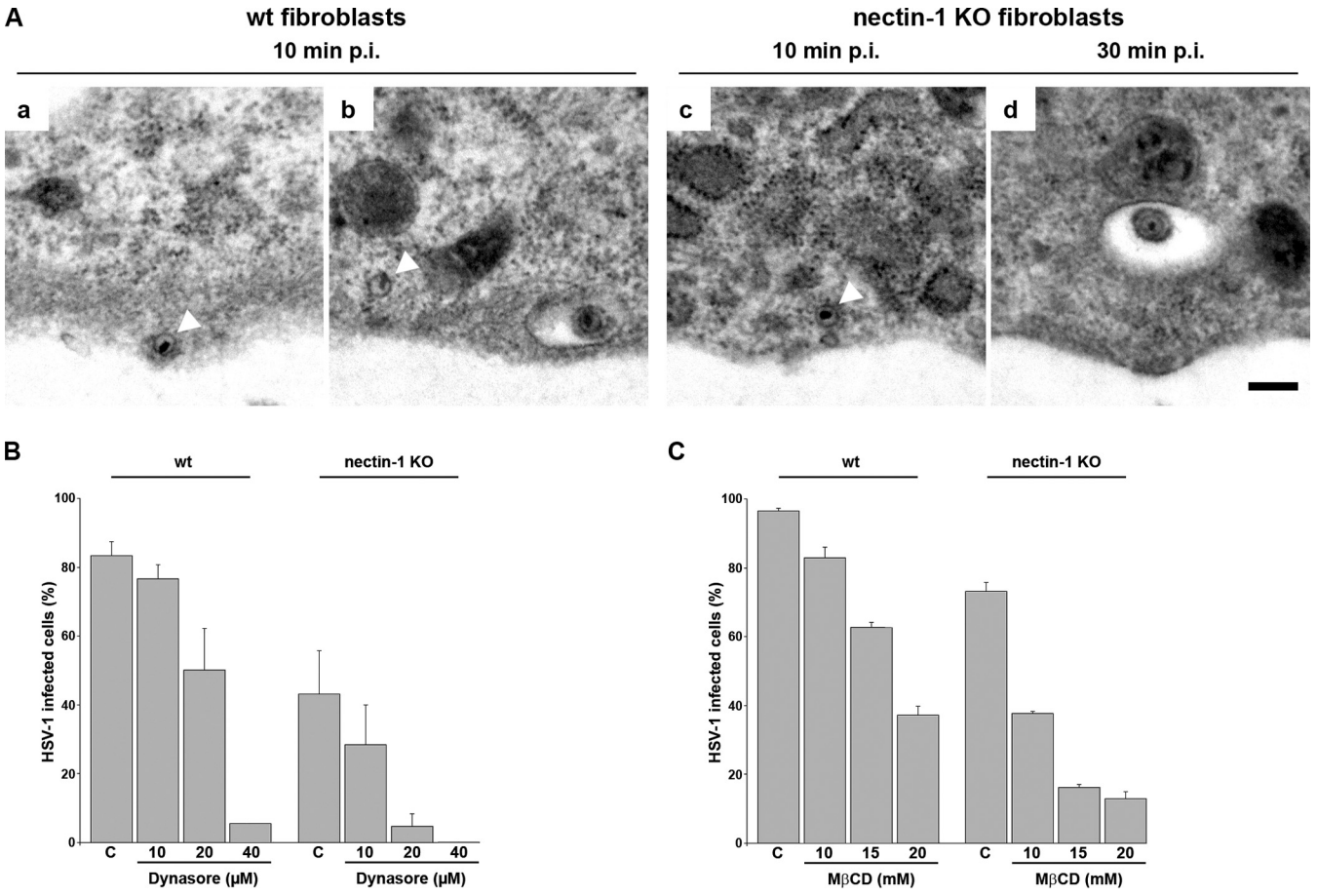

FIG 6 Characterization of HSV-1 uptake into murine wt or nectin-1-deficient primary dermal fibroblasts. (A) EM analyses of wt or nectin-1-deficient primary fibroblasts infected for 10 or 30 min show free cytoplasmic capsids (indicated by arrowheads) (a, b, and c) and enveloped particles in vesicles (b and d). Bar, 0.2 μm. (B) Wild-type or nectin-1-deficient primary fibroblasts were pretreated with DMSO (c = control) or the indicated concentrations of dynasore, followed by infection at 20 PFU/cell. The percentages of ICP0-expressing cells were determined in at least three independent experiments and demonstrate the inhibitory effect of dynasore in both wt and nectin-1-deficient cells. (C) Wild-type or nectin-1-deficient primary fibroblasts were pretreated with water (c = control) or the indicated concentrations of MβCD, followed by infection at 20 PFU/cell for 3 h. The percentages of ICP0-expressing cells were determined in at least three independent experiments and indicate that MβCD decreases the efficiency of infection both in the presence or absence of nectin-1. The results are shown as means ± standard deviations.

were independent of the presence of nectin-1. These results suggest that uptake via nectin-1 and HVEM both involves direct fusion with the plasma membrane and endocytic uptake. Both uptake modes are also observed in murine primary epidermal keratinocytes where nectin-1 acts as the sole receptor, and in murine epidermis where HVEM probably plays a minor role as receptor (14). We assume that uptake via fusion with the plasma membrane leads to productive infection. Whether one or both of these uptake modes can lead to productive infection is difficult to determine, but our previous studies in human keratinocytes support endocytic uptake as contributing to HSV-1 entry (37).

In addition to initial entry of free virus, nectin-1 and HVEM are also considered to function in cell-to-cell spread of virus progeny (39–41). We observed a reduced cell-to-cell spread in nectin-1-deficient dermal fibroblasts, despite the presence of HVEM on all cells. The minor role of HVEM in viral spread is in line with the delayed and reduced virus production seen in nectin-1-deficient fibroblasts. In murine epidermis we also found less efficient spread of HSV-1 to the suprabasal layers in the absence of nectin-1, which correlated with HVEM being present only on a subpopulation of keratinocytes (14). These results support a central role for nectin-1 in efficient cell-to-cell spread of HSV-1 in skin.

In experiments with HVEM-overexpressing cells, exposure to virus resulted in a rapid gD-mediated downregulation of HVEM surface expression, which correlated with virus endocytosis (35). This contrasted with the less efficient downregulation of HVEM from the surface of infected dermal fibroblasts observed here. One possible explanation might be that the earlier experiments relied on the overexpression of human HVEM in a murine melanoma cell line. It is possible that this artificial system failed to reproduce the natural interplay between viral gD, endogenous HVEM, and its natural ligands, resulting in more efficient internalization of HVEM and an alternative mode of uptake. However, the potential role of the four natural ligands—LIGHT, LTα, BTLA, and CD160—of HVEM (42) in murine dermal fibroblasts is still not clear since their expression pattern is unknown.

Interestingly, we observed entry into dermal fibroblasts that were deficient in both nectin-1 and HVEM, although the infection efficiency was greatly reduced. The very low infection efficiency might explain why double-KO mice were found to show no signs

of infection with HSV (11–13). Since the 3-OS-HS generating 3-OST-3 enzyme (10) was detected in murine dermal fibroblasts, we suggest 3-OS-HS as a potential candidate to act as a rather inefficient receptor for HSV-1 on these cells. Infection studies in human corneal fibroblasts revealed a role for 3-OS-HS in mediating infection with HSV-1 (27). However, it remains to be shown how efficient this receptor acts on human corneal fibroblasts.

In conclusion, comparison of the two major resident cell types of murine skin, keratinocytes in the epidermis and dermal fibroblasts in the underlying dermis, demonstrated that nectin-1 is less highly expressed on fibroblasts than on keratinocytes. In contrast, HVEM is present on nearly all fibroblasts but only expressed on a few keratinocytes in the epidermis. Irrespective of the expression levels, we present evidence supporting nectin-1 as the major mediator of HSV-1 entry into dermal fibroblasts and epidermal keratinocytes. It is becoming more evident that although HVEM can serve as viral receptor, its interaction with viral gD also modulates cytokine signaling and, hence, the immune response by interfering with the natural ligands and that this may be its major contribution during infection of the human host (43, 44).

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