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Role of AF6 protein in cell-to-cell spread of Herpes simplex virus 1

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Abstract AF6 and its rat homologue afadin are multidomain proteins localized at cell junctions and involved in intercellular adhesion. AF6 interacts via its PDZ domain with nectin-1 at epithelial adherens junctions. Nectin-1 serves as a mediator of cell-to-cell spread for Herpes simplex virus 1 (HSV-1). We analyzed the role of AF6 protein in the viral spread and nectin-1 clustering at cell–cell contacts by knockdown of AF6 in epithelial cells. AF6 knockdown reduced efficiency of HSV-1 spreading, however, the clustering of nectin-1 at cell–cell contacts was not affected. Thus, AF6 protein is important for spreading of HSV-1 in epithelial cells, independently of nectin clustering, possibly by stabilization of the E-cadherin-dependent cell adhesion.

Keywords: AF6; Herpes simplex virus 1; Cell-to-cell spread; Nectin; PDZ

1. Introduction

AF6 protein has been first described as a fusion partner of the acute lymphoblastic leukemia gene ALL-1, which arises by a chromosomal translocation t(6;11) (ALL-1 Fusion partner from chromosome 6) [1]. It is a multidomain protein harboring one PSD-95/Dlg-1/ZO-1 domain (PDZ), two Ras associating domains (RA), a forkhead-associated domain (FHA), a dilute domain (DIL), and two proline rich regions (pro) [1–3]. Three different splicing variants of human AF6 protein are known, termed isoform 1, 2, and 3 (AF6i1, 2 and 3) [4]. For the AF6 rat homologue afadin, two splicing variants have been reported, termed l-afadin for the long transcript and s-afadin for the short transcript [5]. AF6i3 protein and l-afadin harbor an F-actin binding site at their C-termini [5,6].

AF6 is localized at adherens junctions in epithelial cells and at cell–cell contacts in non-epithelial cells [5,7]. It plays an important role in cell–cell adhesion and development [8,9], as well as in the negative regulation of signaling via the small GTPases Ras and Rap1 [10,11]. At adherens junctions, AF6 interacts via its PDZ domain with the C-terminal four amino acids of nectins [12,13]. Nectins are intercellular adhesion molecules and members of the immunoglobulin superfamily [14]. In addition to the nectin/AF6 adhesion system, epithelial adherens junctions also harbor the E-cadherin/catenin adhesion system [15]. The two systems are connected to each other via several proteins and via the actin cytoskeleton [3]. Recently, it has been shown that AF6 stabilizes the E-cadherin-dependent cell–cell adhesion [6,16].

The alphaherpesvirus Herpes simplex virus 1 (HSV-1) infects damaged mucosal epithelial cells during primary infection and spreads from there to the neurons, where latency is established [17]. Attachment and entry of HSV-1 into the cell requires cellular receptors, including the herpes virus entry mediator (HVEM) [18], 3-O-sulphated heparan sulfate [19], and nectin-1 [20]. All these cellular entry receptors interact with the viral glycoprotein D (gD) [21]. Once entered into the cell, HSV-1 spreads very efficiently from cell-to-cell at areas of cell–cell contacts [22,23], in a space where it is protected from the virus-neutralizing antibodies by tight junctions (TJ), the apical-most cell–cell contact in polarized epithelial cells [24].

Nectin-1 serves not only as a cellular entry receptor for HSV-1, but also as a mediator of the viral cell-to-cell spread [22,25–27]. Two independent studies investigated the role of the interaction between AF6 and the cytoplasmic domain of nectin-1 in this process. Sakisaka and co-workers compared the full-length nectin-1 and a deletion mutant of nectin-1 lacking the four C-terminal amino acids (nectin-1-ΔC), which is unable to interact with AF6/afadin [27]. They reported the necessity of the nectin-1 C-terminus for an efficient viral cell-to-cell spread in fibroblasts. By contrast, Krummenacher and co-workers reported that nectin-1 with a C-terminal GFP tag, which blocks the binding to AF6, was as efficient in supporting viral cell-to-cell spread as the wild-type nectin-1, using B78H1 melanoma cells [26]. Consistently, Even and co-workers showed that the nectin-1 cytoplasmic domain is not required for the cell-to-cell spread of HSV-1 using the same cell system [25]. Because of these contradicting reports, we considered the possibility that AF6 has a function in viral cell-to-cell spread that is independent of its interaction with nectin-1. We addressed the role of AF6 directly by generating small-hairpin RNA (shRNA)-mediated AF6 knock-down in non-transformed human epithelial cells. By that, we show that AF6 does not influence the clustering of nectin-1 at epithelial cell–cell contacts. However, AF6 plays an important role for an efficient cell-to-cell spreading of HSV-1, possibly by stabilizing the E-cadherin-dependent intercellular adhesion.

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2. Materials and methods

2.1. Cell culture, generation of stable cell lines and Western blot analysis

HEK293T, HeLa, and Vero cells were grown in DMEM supplemented with 10% FCS and MCF10A cells (ATCC, Rockville, MD) as previously described [28].

MCF10A cells stably expressing shRNA were generated via retroviral transduction. Recombinant retroviruses were obtained by cotransfection of HEK293T cells with the shRNA-encoding pMSCVpuroΔ3′LTR [6], pVPack-GP and pVPack VSV-G (Stratagene), using Lipofectamine 2000™ (Invitrogen). Following transduction, stably transduced cells were selected with 2 μg/ml puromycin for 10 days. Pooled polyclonal cell populations were used in all experiments.

AF6 knock-down cells stably expressing the reconstituted AF6ΔPDZ construct (pooled polyclonal populations) were generated by lentiviral transduction without selection (transduction efficiency >90%). Recombinant lentivirus was obtained from cotransfection of HEK293T cells with pFUW plasmid encoding AF6ΔPDZ, pCMV-ΔR 8.2 [29,30] and pVPack VSV-G. The Lentiviral expression plasmid harboring the AF6ΔPDZ coding region has been previously described [6]. AF6ΔPDZ protein lacks the aa 998–1062 of AF6 (U02478) and the cDNA construct harbors six silent point mutations between nucleotides 210 and 225 of AF6 ORF, in order to resist the degradation by AF6 shRNA.

For Western blot analysis the cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5% Triton-X-100, 0.5% SDS, 0.1% DOC, 0.5 mM DTT, 10 mM Na_3VO_4, 0.5 M NaF, 25 mM β-glycerolphosphate, trasylol, and Complete protease inhibitor cocktail (Roche)). Equal protein amounts were loaded on the denaturing polyacrylamide gel. The detection was performed with monoclonal anti-AF6 antibody, clone 35 (Transduction Laboratories).

2.2. Plaque assay and determination of viral titers

Cell-to-cell spread of HSV-1 was quantified in a plaque assay. MCF10A cells were grown on 35-mm dishes containing a glass cover slip (diameter 18 mm) until confluency was reached and subsequently incubated with 100 plaque forming units HSV-1 particles per well for 60 min at 37 °C. Monolayers were washed, overlaid with DMEM supplemented with 2% FCS and 0.4% Noble agar and incubated for different periods of time (12 h, 14 h, and 20 h) at 37 °C. For determining the number and size of plaques the coverslips were removed from the wells and plaques were visualized by immunofluorescence. To quantify the viral spread, plaques were counted and grouped according to their size into large (>200 cells), medium (15 to approximately 200 cells) small (4–15 cells), and very small (1–3 cells) plaques.

For determination of viral titers, the cells were grown and infected as described for the plaque assay without the agar overlay. Supernatants were collected at different time points and used at different dilutions to infect Vero cells and determine the number of plaque forming units per volume of MCF10A supernatant.

2.3. Immunofluorescence microscopy

Cells grown on glass coverslips or in tissue culture plates were fixed with 4% PFA for 5 min and permeabilized with 0.5% Triton-X-100 for 5 min. Stained cells were mounted in mounting medium containing 0.2 g/ml Mowiol 4-88 (Calbiochem).

For the visualization of viral plaques, phase contrast and immunofluorescence images were acquired with 4×/NA 0.1 air objective, using inverted microscope (DMIL, Leica), equipped with Leica DC 350FX digital camera. Digital images were acquired with Leica IM50 Image Manager software (Leica). All other immunofluorescence images were obtained using Leica confocal system TCS SP2 (Leica) and a microscope (DMIRBE, Leica) equipped with a 40×/ NA 1.25 oil immersion objective. Digital images were obtained using Leica confocal software 2.61 (Leica).

FITC-coupled murine anti-HSV-1-gC antibody was from Dade Behring AG, Düdingen, Switzerland. Anti-AF6 [31] and anti-nectin-1 CK41 antibody [32] have been previously described. FITC or TRITC-labelled secondary antibodies were from Jackson ImmunoResearch Laboratories Inc.

3. Results

3.1. AF6 knock-down affects cell-to-cell spreading of HSV-1

The role of AF6 protein in the cell-to-cell spread of HSV-1 was analyzed in human mammary epithelial MCF10A cells by AF6 knock-down. Polyclonal cell lines stably expressing shRNA against AF6 (AF6 shRNA) or a control shRNA were generated. The specific downregulation of AF6 protein expression in MCF10A AF6 knock-down cells was analyzed by Western Blotting of whole cell lysates. Expression of AF6 shRNA resulted in approximately 90% reduction of endogenous AF6 protein level (Fig. 1).

We chose the MCF10A cell line for our study because of its non-transformed epithelial character. Confluent monolayers of these cells showed epithelial morphology with a prominent cortical actin cytoskeleton (data not shown) and predominant localization of junctional protein AF6 (Fig. 1B), nectin-1 (Fig. 3B), E-cadherin, α- and β-catenin, and claudin-1 to cellular junctions (data not shown).

In order to analyze cell-to-cell spreading of HSV-1, confluent monolayers of AF6 knock-down and control cells were infected with equal amounts of virus particles. Viral plaques were visualized by immunostaining with anti-HSV-1-gC antibody (Fig. 2A). The size of viral plaques at different time points was determined and the plaques were grouped according to their size in very small, small, middle, and large. Criterion for the plaque size was the number of HSV-infected cells. Very small plaques contained 1–3 infected cells, small plaques 4–15 infected cells, medium size plaques 15 to approximately

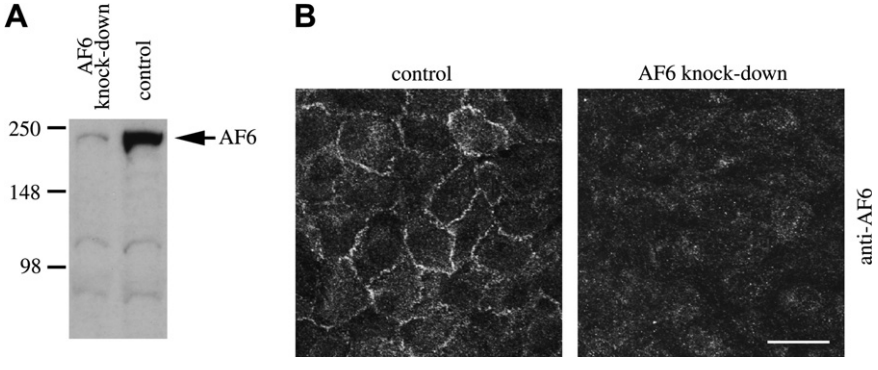

Fig. 1. Small-hairpin RNA (shRNA)-mediated knock-down of AF6 protein in epithelial MCF10A cells. (A) Western blot analysis of whole cell lysates from MCF10A control and AF6 knock-down cells, detected with anti-AF6 antibody. (B) Immunofluorescence pictures of confluent cell monolayers of control and AF6 knock-down cells, using anti-AF6 antibody. Bar, 15 μm.

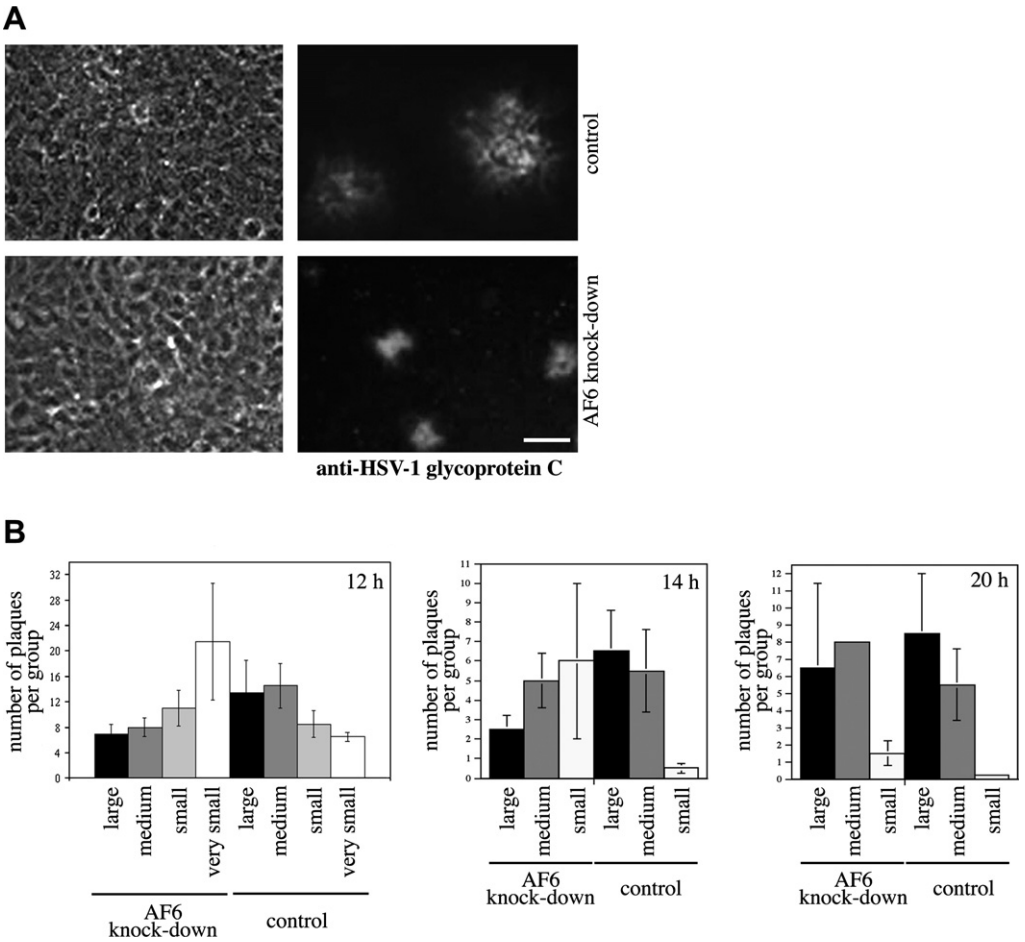

Fig. 2. Analysis of the HSV-1 cell-to-cell spread. (A) Light microscopy (left) and immunofluorescence pictures (right) of confluent cell monolayers during the plaque assay, 14 h after infection of the cells with HSV-1. Viral plaques were visualized by immunostaining with anti-HSV-1 glycoprotein C antibody. Images are representative of three independent experiments. Bar, 100 μm. (B) Graphic representation of the viral plaque size 12 h (left panel), 14 h (middle panel), and 20 h (right panel) after HSV-1 infection. Viral plaques were measured and ordered into four groups according to their size. The experiments shown are representative for 2 (12 h time point) and 4 (14 and 20 h time points) experiments showing similar data. Bars represent S.D.

200 cells, and large plaques >200 cells. At 12 h post infection the number of large and medium size plaques was strongly reduced in AF6 knock-down cells whereas the number of very small plaques was strongly increased when compared to control cells (Fig. 2B, left panel). Fourteen hours post infection the number of small plaques found in monolayers of AF6 knock-down cells was still significantly increased and the number of large plaques significantly decreased as compared to the control cells (Fig. 2A and 2B, middle panel). At this time point, very small plaques were no longer detectable (Fig. 2B). However, at 20 h post infection, the number of plaques and their sizes were similar for AF6 knock-down and control cells (Fig. 2B, right panel). Thus, cell-to-cell spreading of HSV-1 progressed in its initial phase more slowly in AF6 knock-down cells. Importantly, no difference in number of plaques between the AF6 knock-down and control cells was observed, showing that the reduction of AF6 expression did not alter the infectivity of MCF10A cells. We also did not observe any difference in the cytopathic effect between the two cell lines.

The growth kinetic of HSV-1 in MCF10A AF6 knock-down and control cells was compared. Both cell lines were infected with equal amounts of infectious HSV-1 particles for 45 min at room temperature prior to addition of fresh medium. Cell culture supernatants were collected at different time points after infection and virus titers were determined on Vero cells by serial dilutions. Viral titers obtained for AF6 knock-down and control cells were identical (1×10^4 pfu/ml) at 14 h post infection (Fig. 3A), showing that the growth kinetic of HSV-1 was not affected by AF6 knock-down. Therefore, the reduced size of viral plaques in AF6 knock-down cells 14 h post infection was indeed due to the reduced viral cell-to-cell spread. At 20 h post infection the viral titer was one log lower in AF6 knock-down cells as compared to the control (Fig. 3A), but again reached the same level as in the control cells at 36 h post infection (data not shown).

3.2. Analysis of nectin-1 and AF6 clustering at cell–cell contacts

Deletion of the four C-terminal amino acids of nectin-1 (nectin-1-ΔC) reduced its localization at cell–cell contacts and resulted in a reduced cell-to-cell spread of HSV-1 [27]. The interaction between AF6 and nectins has been reported to be important for their efficient clustering at cell–cell junctions in fibroblasts [12,13]. Therefore, AF6 knock-down might reduce the clustering of nectin-1 at cell–cell contacts and thereby affect

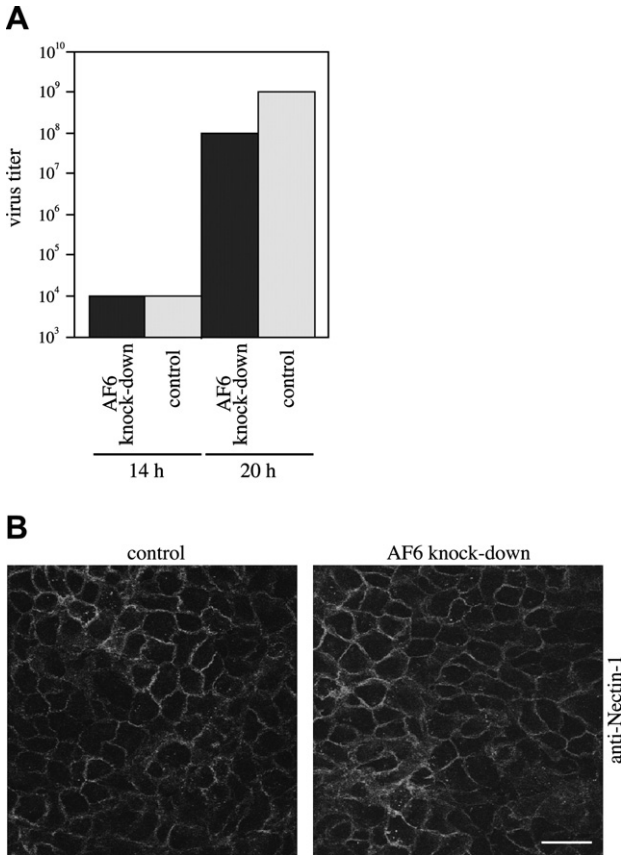

Fig. 3. Growth kinetic of HSV-1 in confluent monolayers of MCF10A cells. Comparison of the virus titers (plaque forming units per ml) in the supernatant of AF-6 knock-down and control cells 14 and 20 h after HSV-1 infection. Individual columns represent the mean values of three independent experiments. S.D. are $\leqslant 1\%$ of the indicated virus titer. (B) Localization of nectin-1 at cell junctions in MCF10A cells. Immunofluorescence staining of confluent monolayers of AF6 knock-down and control cells, performed with anti-nectin-1 antibody. Bar, 30 μm.

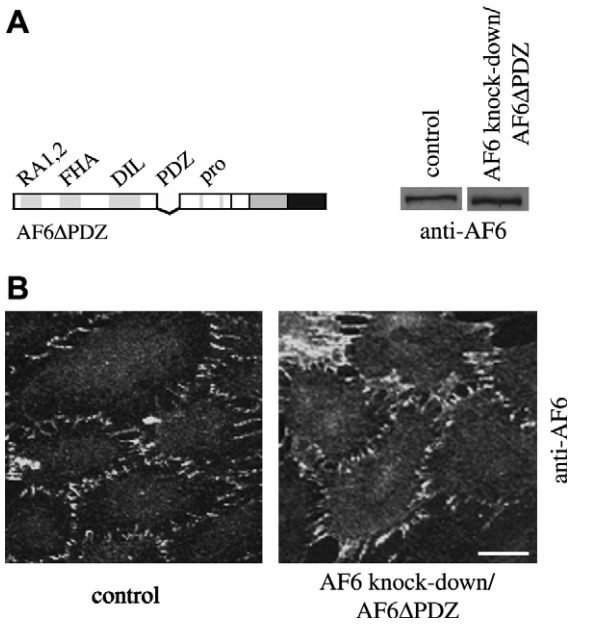

Fig. 4. Reconstitution of AF6 knock-down cells with AF6 protein lacking the PDZ domain (AF6ΔPDZ). (A) Schematic representation of AF6 with the deletion of the PDZ domain. Western blot analysis of whole cell lysates from control cells and AF6 knock-down cells expressing AF6ΔPDZ protein. The detection was performed with anti-AF6 antibody. (B) Immunofluorescence analysis of control cells and AF6 knock-down cells expressing AF6ΔPDZ protein. Staining was performed with anti-AF6 antibody. Bar, 5 μm.

the viral cell-to-cell spread. To test this, we performed immunofluorescence analysis of confluent monolayers of AF6 knock-down and control cells with anti-nectin-1 antibody. In both cases, nectin-1 localized efficiently at cell–cell junctions and no difference was observed between AF6 knock-down and control cells (Fig. 3B). Thus, AF6 did not act on HSV-1 spread through nectin-1 clustering, since this was independent of AF6 in epithelial MCF10A cells.

Although the localization of nectin-1 at cell–cell contacts was independent of AF6, the localization of AF6 might be dependent on nectin-1 or other members of the nectin family, as has been previously reported for non-epithelial or transformed cells [12,26,27]. AF6 has been shown to interact with the C-terminus of nectins via its PDZ domain [13]. To test whether AF6 is recruited to cell–cell contacts in nectin-dependent manner, we constructed an AF6 deletion mutant lacking the PDZ domain (AF6ΔPDZ). This construct was made resistant to shRNA-mediated degradation by introduction of six silent point mutations within the targeted region and stably expressed in AF6 knock-down cells. Confluent cell monolayers of control cells expressing endogenous full-length AF6 protein and monolayers of AF6 knock-down cells reconstituted with AF6ΔPDZ protein were stained with anti-AF6 antibody and analyzed by immunofluorescence. No difference in the clustering of full-length AF6 and the AF6ΔPDZ protein at cell–cell contacts could be detected (Fig. 4). Thus, the localization of AF6 protein at cell–cell contacts in confluent monolayers of MCF10A cells was independent of the AF6-PDZ-mediated interaction with nectins.

4. Discussion

HSV-1 is an alphaherpesvirus, which infects damaged mucosal epithelial cells during primary infection and disseminates from there to neurons by spreading from cell-to-cell across the cell junctions. The HSV-1 receptor nectin-1 and its interaction partner AF6 are both localized to adherens junctions. While it is known that nectin-1 is required for efficient viral cell-to-cell spread, the role of AF6 is not clear [25–27]. Here, we directly addressed the role of AF6 protein in the viral cell-to-cell spread in human epithelial cells by AF6 knock-down, and demonstrate that AF6 plays an important role in this process.

4.1. AF6 knock-down affects cell-to-cell spreading of HSV-1

Knock-down of AF6 expression had no effect on the total number of viral plaques, demonstrating that AF6 is not essential entry of HSV-1. We observed a slightly reduced viral titer in the supernatant of AF6 knock-down cells when compared to controls at 20 h post infection. This is most likely a consequence of a slower viral spread early after infection and hence a slightly lower production of virus particles. The fact that we

did not observe a difference in viral titers between these two cell lines at an earlier time point (14 h p.i.) may be explained by the low titer (see Fig. 3).

Our data demonstrate that downregulation of AF6 expression by RNAi (Fig. 1) lead to an impaired cell-to-cell spread of HSV-1 at early time points post infection (Fig. 2B). Later in infection (20 h post infection), however, the influence of AF6 on HSV-1 spreading was no longer evident. The reason for this transient effect of AF6 remains to be elucidated. Two earlier studies addressing the role of AF6 on HSV-1 spreading indirectly by overexpressing nectin-1α mutants with either deleted or masked C-terminal PDZ binding revealed conflicting results [26,27]. The first study using C-terminally truncated nectin-1α in non-epithelial L cells, showed a reduced cell-to-cell spread of HSV-1 [27] strongly suggesting that efficient viral cell-to cell spread depends on an interaction between nectin-1α and AF6. By contrast, the second study using a C-terminally GFP-tagged nectin-1α, thus preventing AF-6 binding, did not reveal any reduction in viral plaque size in epithelial melanoma cells [26]. Moreover, recently Even and coworkers reported that the extracellular V-like domain of nectin-1, but not its cytoplasmic domain containing the C-terminal PDZ ligand is required for efficient cell-to-cell spread of HSV-1 [25], suggesting that PDZ-mediated interaction of nectin-1α and AF6 is not required. These discrepancies may be explained by differences in the composition of junctional protein complexes at cell–cell contacts of the different cell lines used. For instance, it has been demonstrated that the E-cadherin-catenin adhesion system increases the efficiency of HSV-1 cell-to-cell spread [27]. In epithelial MCF10A cells knockdown of AF6 resulted in impaired E-cadherin-dependent intercellular adhesion [6], which may explain the reduced cell-to-cell spread of HSV-1 in our study. Consistently, L cells not expressing E-cadherin also showed reduced cell-to-cell spread compared to L cells ectopically expressing E-cadherin (EL cells) [27]. It is also conceivable that AF6 exerts its function in viral cell-to-cell spread independent of receptors at other sites than cell junctions, since AF6 is not only located at cell–cell contacts but also in the cytoplasm [6].

4.2. Analysis of nectin-1 and AF6 clustering at cell–cell contacts

The question whether accumulation of nectin-1 at cell junctions requires interaction with AF6 is also a controversial issue. Initially, two studies suggested that AF6 is required for the recruitment of nectin-1 to cell–cell contacts [12,13]. Two later reports, however, showed that nectin-1 localizes to cell contact areas independent of an interaction with AF6 [26,27]. Our data showing that in cells with downregulated AF6 expression, nectin-1 still localized to cell–cell contacts (Fig. 3B) confirm the observations of the two latter studies.

Since we used a nectin-1 specific antibody directed against its extracellular V-domain we cannot exclude the possibility that in AF6 knock-down cells we primarily detect nectin-1 isoforms (e.g. nectin-1β) at cell junctions lacking the C-terminal AF-6 interaction sequence.

Several previous studies using nectin-1α mutants with either masked or deleted PDZ binding site suggested that AF6 is recruited to cell–cell contacts by nectin-1 via its PDZ ligand [13,26,27]. However, we observed that expression of an AF6 mutant lacking the PDZ domain still localized to cell–cell junctions in MCF10A cells. Hence, AF6 interaction with members of the nectin family via its PDZ domain is not essential for its localization. Possible explanations for this finding are that in MCF10A cells AF6 may be recruited to cell–cell contacts by association to nectin via a second, PDZ independent binding site or in a nectin-independent manner, e.g. through interaction with other junctional proteins such as ZO-1 or α-catenin [31,33,34].

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