The Avian Influenza Virus NS1 ESEV PDZ Binding Motif Associates with Dlg1 and Scribble To Disrupt Cellular Tight Junctions[∇]§

Lisa Golebiewski, Hongbing Liu, Ronald T. Javier, and Andrew P. Rice*

Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030

Received 12 May 2011/Accepted 2 August 2011

The influenza A virus NS1 protein contains a conserved 4-amino-acid-residue PDZ-ligand binding motif (PBM) at the carboxyl terminus that can function as a virulence determinant by targeting cellular PDZ proteins. The NS1 proteins from avian and human viral isolates have consensus PBM sequences ESEV and RSKV, respectively. Currently circulating highly pathogenic H5N1 viruses contain the ESEV PBM which specifically associates with the PDZ proteins Scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3. In this study, we found NS1 proteins from viral isolates that contain the PBM sequence RSKV, KSEV, or EPEV are unable to associate with these PDZ proteins. Other results showed that the ESEV PBM mediates an indirect association with PDZ protein, Lin7C, via an interaction with Dlg1. Infection with a virus that expresses a NS1 protein with the ESEV PBM results in colocalization of NS1, Scribble, and Dlg1 within perinuclear puncta and mislocalization of plasma membrane-associated Lin7C to the cytoplasm. Infection of polarized MDCK cells with the ESEV virus additionally results in functional disruption of the tight junction (TJ) as measured by altered localization of TJ markers ZO-1 and Occludin, decreased transepithelial electrical resistance, and increased fluorescein isothiocyanate (FITC)-inulin diffusion across the polarized cell monolayer. A similar effect on the TJ was observed in MDCK cells depleted for either Scribble or Dlg1 by small interfering RNA (siRNA). These findings indicate that ESEV PBM-mediated binding of NS1 to Scribble and Dlg1 functions to disrupt the cellular TJ and that this effect likely contributes to the severe disease associated with highly pathogenic H5N1 influenza A viruses.

The epithelial barrier is an important defense against viral infection and is maintained by tight junction (TJ) proteins that limit transport across the epithelia. TJs also serve to separate apical and basolateral compartments of the epithelial barrier (57). TJ-associated proteins are attractive targets for viruses, as TJ disruption allows access to receptors for viral entry, dissemination in the infected host, and enhanced spread between hosts (for a review, see reference 15). Reovirus, coxsackievirus, and hepatitis C virus (HCV) all utilize TJ proteins as receptors for viral entry (2, 8, 11). Rotavirus disrupts localization of the TJ protein ZO-1 through the action of the NSP4 toxin, and the rotavirus VP8 core protein blocks the formation of tight junctions (35, 50). Adenoviruses utilize TJ proteins for internalization and release (58). Rhinovirus replication within polarized primary human airway epithelial cells disrupts transepithelial electrical resistance (TER), a measure of TJ integrity, through downregulation of both protein and mRNA levels for ZO-1 and the adherens junction (AJ) component E-cadherin (43, 62). The HIV-1 gp120 protein is able to inhibit localization of ZO-1, Occludin, and claudins to the TJ, contributing to a disrupted intestinal mucosa during HIV-1 infection (36).

The influenza A virus nonstructural protein 1 (NS1) is a key virulence factor for infection, and it functions to inhibit the host innate immune response through the association with a

number of antiviral proteins (reviewed in reference 16). Additionally, a large-scale sequencing project identified PDZ-binding motifs (PBMs) located at the last four amino acid residues of the NS1 protein (40). PBMs confer binding to a class of cellular proteins that contain a characteristic structure known as the PDZ domain. In general, PDZ domain-containing proteins act as scaffolds to assemble protein complexes and function in cell signaling and cellular polarity (reviewed in references 17 and 39). From 2000 to 2011, NS1 proteins from human influenza A virus isolates generally contain a PBM with the sequence RSKV in H3N2 viruses (~97%) and RSEV in H1N1 viruses (~88%), excluding the 2009 swine origin pandemic H1N1 viruses which encode an NS1 protein with deletion of the PBM. NS1 proteins from H5N1 avian influenza A virus isolates from human infections generally have a PBM with the sequence ESEV (~79% of isolates) (1). The currently circulating highly pathogenic H5N1 influenza viruses generally contain a PBM with the sequence ESEV, with the notable exception of a clade from 1997 H5N1 viruses, where NS1 proteins typically had a noncanonical EPEV PBM (40). Since 2000, this subset of NS1 proteins containing the EPEV PBM has become relatively rare in the H5N1 NS1, representing less than 3% of avian origin and less than 1.5% human origin H5N1 NS1 PBMs (1). The human and avian PBM sequences RSKV, RSEV, and ESEV are a canonical class I PBM with the consensus sequence -X-S/T-X-V_{COOH} and are predicted to bind several PDZ proteins (39, 40, 45, 55).

Mouse studies with recombinant H1N1 human influenza viruses found that NS1 proteins with the PBM sequence ESEV, EPEV, or RSKV enhance viral replication and pathogenesis (23). However, recent studies have suggested that the PBM's contribution to pathogenesis may be species specific.

* Corresponding author. Mailing address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX 77030. Phone: (713) 798-5774. Fax: (713) 798-3490. E-mail: arice@bcm.tmc.edu.

§ Supplemental material for this article may be found at <http://jvi.asm.org/>.

[∇] Published ahead of print on 17 August 2011.

The RSKV PBM enhanced virus replication in duck and human cells, whereas the ESEV PBM increased virulence in mice (46). Another study found the ESEV PBM attenuated virus replication in mammalian and duck cells but did not alter virus fitness in chicken cells (65). Protein binding studies have shown that the avian ESEV consensus PBM can specifically associate with the PDZ proteins Scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3 (32, 52). The association between the NS1 ESEV PBM and Scribble is a direct protein-protein interaction that functions to inactivate Scribble's proapoptotic function and to enhance influenza A virus replication (32). The functional significance of the interaction between NS1 with an ESEV PBM and Dlg1, MAGI-1, MAGI-2, and MAGI-3 is not known. It is also not known which cellular PDZ proteins are targeted by the RSKV PBM or the noncanonical EPEV PBM. Recent studies also reported that the NS1 ESEV PBM confers binding to a PDZ protein named PDLim2 and PSD-95 (63, 64). PDLim2 is expressed in epithelial cells and lymphocytes and functions to negatively regulate NF- κ B; the functional significance of the interaction between NS1 and PDLim2 is not known (63). PSD-95 is highly expressed in neuronal cells and acts as a scaffolding protein at postsynaptic sites for cytoskeleton components, neurotransmitter receptors, and adhesion molecule ionic channels (25). The association of PSD-95 and NS1 in neuronal cells inhibits the production of nitric oxide, which may enhance virus replication (64).

In this study, we sought to identify cellular PDZ proteins that bind various canonical and noncanonical PBMs found in human and avian influenza viruses. Our PDZ screen was unable to identify any PDZ associations with the RSKV, KSEV, or EPEV PBM. We confirmed that an H5N1 NS1 with the ESEV PBM associated with the previously identified PDZ proteins MAGI-1, MAGI-2, MAGI-3, Scribble, and Dlg1. We also found that NS1 with an ESEV PBM mediated a novel indirect association with Lin7C through the NS1 interaction with Dlg1. During infection of recombinant viruses encoding the NS1 ESEV motif, NS1 colocalized with Dlg1 and Scribble in perinuclear puncta and mislocalized Lin7C. Furthermore, ZO-1 and Occludin were mislocalized from the TJ in cells infected with ESEV virus. Small interfering RNA (siRNA) depletion of Dlg1 or Scribble also disrupted the TJ integrity. During infection with the ESEV virus, infected monolayers displayed a decrease in TJ integrity and an increase in paracellular permeability over the course of infection with ESEV virus. Our results indicate that the ESEV PBM allows NS1 to disrupt the host TJ and to increase permeability of infected monolayers by the inactivation of Dlg1 and Scribble.

MATERIALS AND METHODS

Cells and cell extracts. 293T, A549, and MDCK cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Invitrogen and Sigma) at 37°C with 5% CO₂. Cells were lysed with EBC buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol [DTT]) containing protease inhibitor cocktail (Sigma) and prepared as described previously (18).

Plasmids and recombinant protein purification. NS1 proteins used in this study are described in Table S1 in the supplemental material. Plasmids encoding the NS1 gene from an H6N6 avian influenza A virus isolate (A/blue-winged teal/MN/993/1980) and an H3N2 human influenza A virus isolate (H3N2 A/Memphis/14/1998) were provided by Clayton Naeve (40); the NS1 from an H5N1 human influenza A virus isolate (A/Viet Nam/1194/2004) and an H5N1 avian influenza A virus isolate (A/Hong Kong/156/97) were kindly provided by

Yoshiro Kawaoka (University of Wisconsin) and Peter Palese (Mount Sinai School of Medicine), respectively. These plasmids were used as PCR templates to create *Escherichia coli* and mammalian expression plasmids. The NS1 effector domains (ED) were fused to glutathione *S*-transferase (GST) to create GST-NS1 expression plasmids (pGEX-2T). The H3N2, H6N6, and avian H5N1 ED contain residues 73 to 230, whereas the human H5N1 ED has a 5-amino-acid-residue truncation within the linker region, between the RNA binding domain (RBD) and ED, and contains residues 73 to 225 (6). GST-NS1 fusion proteins were expressed and purified as previously described (18). Mammalian constructs consisted of full-length NS1 fused to the FLAG epitope tag (pFLAG-CMV). To allow increased expression levels of NS1 proteins from the expression plasmids, the CPSF30 binding site at residues 184 to 188 was altered from GLEWN to RFLRY (38). Alterations to the PBM of the H6N6 and H5N1 NS1 proteins were generated with the QuikChange site-directed mutagenesis kit (Stratagene).

PDZ protein mammalian expression proteins were GW1/HA-MAGI-1B, GW1/HA-MAGI-1C (13), pcDNA3/HA-MAGI-2, pcDNA/MAGI-3-V5 (53), GW1/HA-MUPP1 (29), pRK5/Myc-PATJ (27), pcDNA/FLAG-Scribble (34), GW1/HA-Dlg1-I2, GW1/HA-Dlg1-I3, pEGFP-C3-Dlg1- Δ PDZ1, pEGFP-C3-Dlg1- Δ PDZ2, pEGFP-C3-Dlg1- Δ PDZ3, pEGFP-C3-Dlg1- Δ PDZ1-2, and pEGFP-C3-Dlg1- Δ 3PDZ (12). To construct a mammalian expression vector for Lin7C, PCR was used to insert the coding region of Lin7C (Origene) into a histidine-tagged expression vector (pET46; Novagen). Expression and purification of His-Lin7C were carried out according to the manufacturer's protocol (Qiagen).

GST-NS1 binding assays. 293T cells were transfected at 50% confluence with epitope-tagged PDZ protein expression plasmids with Lipofectamine and Plus reagent (Invitrogen). Cell extracts were prepared 24 h posttransfection, and binding assays with GST-NS1 proteins were performed as previously described (32).

siRNA, immunoprecipitations and immunoblots. Small interfering RNA (siRNA) targeting human Scribble (51) with the target sequence 5'-CAGGATGAAGTCATTGGAACA-3' was purchased from Ambion. siRNA targeting human Dlg1 was purchased from Santa Cruz. Canine Scribble and canine Dlg1 siRNA used the following target sequences: 5'-GGGAAGATGGCGAGAGCGA-3' and 5'-GAGAAGAACCUGUCAGAAA-3' (Dharmacon), respectively. 293T cells were reverse transfected with siRNA targeting human Dlg1 or Scribble with RNAiMAX according to the manufacturer's instructions. Protein depletion was confirmed at 48 h posttransfection by immunoblotting.

293T cells were transfected with FLAG-NS1 plasmids with Lipofectamine and Plus reagent according to the manufacturer's protocol, and extracts were prepared at 24 h posttransfection. Cell extracts were incubated with anti-FLAG M2 affinity gel (Sigma) for 1 h, and NS1 complexes were then washed with TBS (50 mM Tris [pH 7.4], 150 mM NaCl) and resuspended in SDS-PAGE loading buffer. Immunoblotting was performed as previously described (20). Antibodies used were to Myc (Cell Signaling), green fluorescent protein (GFP) (Clontech), β -actin, FLAG (M2, Sigma), hemagglutinin (HA), Scribble, Dlg1 (Santa Cruz), p85 β (Serotech), V5, and Lin7C (Invitrogen).

Influenza A viruses. Previously characterized influenza A viruses encoding the H6N6 NS segment (A/blue-winged teal/MN/993/1980) in a Udorn (A/Udorn/72) background were used for all infections (32). These viruses expressed H6N6 NS1 with a wild-type ESEV or mutant ESEA PBM. The identities of viral stocks were confirmed by DNA sequencing.

Immunofluorescence. MDCK and A549 cells were grown on glass coverslips in 24-well plates until confluent. Cells were infected with ESEV or ESEA virus at a multiplicity of infection (MOI) of 3 in DMEM, and after 1 h the medium was replaced with DMEM containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin (2 μ g/ml). At 10 h postinfection, the cells were washed twice with ice cold phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 30 min at 4°C. Coverslips were washed with PBS and permeabilized with 0.5% Triton X-100 for 20 min at room temperature. Coverslips were washed with PBS and blocked overnight in 5% nonfat dry milk (NFDM) in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween) at 4°C. Coverslips were again incubated at 4°C overnight with primary antibody dilutions in 2.5% NFDM in TBST, followed by incubation with secondary antibodies conjugated to Alexa Fluor dyes (Invitrogen). The cells were fixed a second time and quenched with sodium borohydride (1 mg/ml) in PEM [80 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), 5 mM EGTA, 2 mM MgCl₂]. The cells were washed with PBS and then stained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS to visualize the nuclei. Coverslips were mounted on glass slides using ProLong Gold antifade reagent and allowed to cure for 24 h. The cells were then imaged by deconvolution microscopy as described previously (19). A series of focal planes (Z-stacks) were captured for each coverslip with the Applied Precision DeltaVision microscope system and deconvolved with the

softWoRx program (Applied Precision). A single focal plane from each Z-stack was further processed in Adobe Photoshop CS3. Alternatively, for transwell filter inserts, filters were imaged on a Carl Zeiss LSM 510 Meta confocal microscope (Carl Zeiss) and processed by LSM 510 image software (Carl Zeiss Inc.) and Adobe Photoshop CS3.

Primary antibodies used were to NP, Scribble, Dlg1 (Santa Cruz), Lin7C, ZO-1, Occludin (Invitrogen), NS1 (a generous gift from Robert Krug, University of Texas, Austin), ZO-3 (Cell Signaling), and NS1 (Immune Tech Corp.).

TER and MDCK siRNA. MDCK cells were seeded on semipermeable transwell filter inserts (0.4- μ m pore size; Costar), and TER was monitored daily. Approximately 5 days after seeding, monolayers were confluent and experienced a peak in TER, indicating polarization. Monolayers were infected with ESEV or ESEA virus at an MOI of 1 in DMEM, and after 1 h the medium was replaced with DMEM containing TPCK trypsin. TER was measured just before infection and at 3 h and 6 h postinfection. To normalize the readings, the TER of an empty well was subtracted from all infected and mock TER measurements. The ESEV- and ESEA-infected TER measurements were then compared to uninfected TER measurements and expressed as percentages of mock TER. Statistical differences between groups were determined by Student's *t* test, and *P* values of <0.05 were considered statistically significant.

To determine the percentage of infected cells at the conclusion of TER measurements, monolayers were fixed and processed for immunofluorescence. Monolayers were stained for NP and Scribble and then scored for the presence of NP.

MDCK cells were reverse transfected by RNAiMAX, following the manufacturer's protocol, with siRNA targeting canine Scribble and Dlg1 (Dharmacon). SiGLO (Dharmacon) and human Scribble siRNA served as a transfection marker and negative control, respectively. The siRNA-transfected cells were grown on transwell filter inserts, and TER was measured daily. Depletion of Scribble and Dlg1 were confirmed by immunofluorescence and immunoblotting.

FITC-inulin paracellular diffusion. MDCK cells were grown to confluence on transwell filter inserts for approximately 5 days. TER was measured daily until cells reached peak TER readings. The cells were infected with ESEV or ESEA virus at an MOI of 1. After a 1-h virus incubation, the apical inoculum was replaced with medium containing fluorescein isothiocyanate (FITC)-inulin (2 mg/ml) in phenol red-free DMEM. Supernatants were removed from the basolateral chamber at 3 and 6 h postinfection and FITC-inulin was measured using a SpectraMax M5 plate reader. Paracellular diffusion is expressed as the percentage of mock diffusion. Statistical significance was determined by Student's *t* test, and *P* values of <0.05 were considered statistically significant.

RESULTS

Identification of a novel interaction between avian influenza NS1 ESEV PBM and Lin7C. We previously used an *in vitro* binding assay to screen an avian virus H6N6 NS1 effector domain (ED) fusion protein, termed GST-avNS1-ESEV, for the ability to associate with several PDZ proteins (32). The H6N6 GST-avNS1 fusion protein with an ESEV PBM associated specifically with the PDZ proteins Dlg1, Scribble, MAGI-1, MAGI-2, and MAGI-3 but not with MUPP1 or PATJ. To identify additional proteins that associate with the avian NS1-ESEV protein, we created a full-length FLAG-tagged H6N6 NS1 expression plasmid, termed avNS1-ESEV. As a specificity control, we constructed a plasmid that expressed NS1 with an alanine substitution in the PBM, termed avNS1-ESEA. These plasmids were transfected into 293T cells, and cell extracts were subjected to anti-FLAG immunoprecipitations. NS1-associated proteins were identified by mass spectrometry (Table 1).

Several proteins coimmunoprecipitated with wild-type avNS1-ESEV but not mutant avNS1-ESEA protein, including Scribble and Dlg1, as expected. The PDZ protein Lin7C also specifically associated with the avNS1-ESEV protein, as well as a number of non-PDZ proteins listed in Table 1. The molecular masses of Scribble and Dlg1 are approximately 8-fold and 5-fold greater, respectively, than that of Lin7C, and therefore

TABLE 1. Mass spectrometry analysis of FLAG-avNS1-ESEV protein complexes^a

Protein	No. of peptides	Presence of a PDZ domain
Scribble	70	Yes
Dlg1	19	Yes
Lin7C	3	Yes
Hypothetical protein LOC57639	6	No
Transcription elongation factor A protein 2 isoform a	3	No
Psoriasin	3	No
EGF-like module-containing mucin-like hormone r	2	No
Stathmin 1	2	No
Basigin isoform 1	2	No

^a Cellular proteins are shown that coimmunoprecipitated with the FLAG-avNS1-ESEV but not FLAG-avNS1-ESEA protein as determined by mass spectrometry. The numbers of peptides identified for each protein are indicated, as well as whether the proteins contain one or more PDZ domains.

it is not surprising that fewer Lin7C peptides were identified than Scribble and Dlg1 peptides. In this study, we chose to focus on NS1's interaction with Dlg1 and Lin7C, as both proteins are found within a complex at the AJ and influence cellular signaling and TJ integrity, two cellular processes of potential significance to influenza A virus replication and pathogenesis (5, 31).

Interaction of avian NS1 ESEV with Dlg1 acts in a cooperative manner to facilitate binding of Lin7C. Dlg1 has key roles in regulating tight junctions, cellular polarity, and cell cycle progression. Lin7C forms a tripartite complex with Dlg1 and MPP7 within the Crumbs complex, and this protein complex is involved in cellular polarity (3, 4, 5, 31, 48). Dlg1 has been previously shown to be a target of the oncogenic adenovirus E4-ORF1, human papillomavirus (HPV) E6, and human T cell leukemia virus type 1 (HTLV-1) Tax proteins. The interaction between these viral proteins and Dlg1 during viral infection leads to the disruption of cellular polarity, perturbation of signaling pathways, or syncytium formation (12, 21, 33, 49, 54). Dlg1 contains three PDZ domains, a SRC homology domain 3 (SH3), an I3 or I2 domain, and a guanylate kinase (GK) homology domain (Fig. 1A).

To determine which Dlg1 PDZ domains are required for an association with the avian NS1, we transfected GFP-Dlg1 deletion plasmids into 293T cells and used cell lysates in binding assays with the H6N6 GST-avNS1 ED protein containing the ESEV PBM (Fig. 1B). Deletion of PDZ domain 1 or 3 alone did not affect binding of H6N6 GST-avNS1-ESEV ED to Dlg1, and deletion of PDZ domain 2 substantially reduced but did not eliminate binding of H6N6 GST-avNS1-ESEV ED to Dlg1, whereas deletion of PDZ domain 1 and approximately half of PDZ domain 2 abolished the interaction. These data suggest that the interaction between the H6N6 GST-avNS1-ESEV ED and Dlg1 involves PDZ domains 1 and 2, which represent a single functional unit where truncated PDZ domain 2 may confer partial PDZ domain 1 binding to H6N6 ED (12, 28, 59). While it has been suggested that the Dlg1 PDZ domain 3 is critical for binding of ESEV containing NS1 to Dlg1, the association was not completely abolished until deletion of the second Dlg1 PDZ domain (52). Possible explana-

FIG. 1. Requirements of Dlg1 and Lin7C for binding to the avian influenza NS1 protein *in vitro*. (A) Domain structure of Dlg1 is shown: three PDZ domains, a SRC homology domain 3 (SH3), an I3 domain, and an inactive guanylate kinase (GK) domain. Deletion mutants of the indicated PDZ domains are shown. (B) The indicated GFP-tagged Dlg1 deletion mutants were transfected into 293T cells, cell extracts were prepared at 24 h posttransfection, and binding assays performed with the GST-NS1 ED containing the ESEV PBM. Products of binding reactions were evaluated by immunoblots using GFP antisera; binding reaction products were also evaluated by Coomassie blue staining shown in the bottom panel. Lanes indicated by “–” represent input in binding reactions; lanes indicated by “+” are products of the binding reactions. (C) 293T cells were transfected with siRNAs targeting Scribble or Dlg1 for 48 h; cultures were then transfected with full-length FLAG-tagged NS1 protein (H6N6) with indicated PBM or were mock transfected. Lysates were prepared at 24 h posttransfection and used in coimmunoprecipitations. Products of immunoprecipitations were analyzed in immunoblots.

tions for this difference may depend upon the Dlg1 constructs used, as the ones used here have an intact C terminus, containing a third of the total Dlg1 protein and the I3, SH3, and GK domains, which may influence PDZ domain availability and presentation for binding.

To characterize the association between NS1 and Lin7C, a His-tagged Lin7C protein was expressed and purified from *E. coli*. Purified wild-type GST-avNS1-ESEV ED and mutant GST-avNS1-ESEA ED proteins were used in *in vitro* binding assays. However, we were unable to demonstrate a specific direct interaction between the recombinant avNS1-ESEV ED and Lin7C proteins, suggesting that the interaction detected in coimmunoprecipitations is indirect (data not shown). Because

Lin7C and Dlg1 are found in the Crumbs complex and associate through the L27 domain (5), we suspected that Dlg1 may allow Lin7C to associate with the avNS1-ESEV protein through an indirect interaction. To test this, 293T cells were first cotransfected with siRNAs that deplete Dlg1 and with a vector that expresses either FLAG-avNS1-ESEV or FLAG-avNS1-ESEA. As a specificity control, siRNAs that deplete Scribble were also included in the experiment. Cell extracts were then immunoprecipitated with an antibody against the FLAG epitope (Fig. 1C). In cells depleted of Dlg1, neither the avNS1-ESEV nor avNS1-ESEA coimmunoprecipitated with endogenous Lin7C as determined in immunoblots. It is likely that the association between Lin7C and Dlg1 occurs through

L27 domains at the N terminus of each protein (5). However, in cells depleted of Scribble, wild-type avNS1-ESEV but not mutant avNS1-ESEA coimmunoprecipitated with endogenous Lin7C. These data indicate that the association between the avNS1-ESEV protein and Lin7C is dependent upon Dlg1, supporting the idea of an indirect interaction between NS1 and Lin7C.

Characterization of canonical and noncanonical NS1 PBM associations with PDZ proteins. We have previously shown that an H6N6 NS1 protein with the ESEV PBM specifically associates with Scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3 *in vitro* (32). To determine if other NS1 PBM sequences also confer binding to these PDZ proteins, we constructed GST-avNS1 fusion proteins with a variety of PBM sequences for use in *in vitro* binding assays. Our previously characterized H6N6 GST-avNS1-ESEV ED protein was used as the ED platform, and PBM sequences from a variety of influenza A virus isolates were appended to the ED C terminus. These PBM sequences were ESEV (H6N6 A/blue-winged teal/MN/993/1980), EPEV (H5N1 A/Hong Kong/156/1997), KSEV (H1N1 A/Brevig Mission/1/1918), and RSKV (H3N2 A/Memphis/14/1998). These fusion proteins were called GST-avNS1-ESEV, GST-avNS1-EPEV, GST-avNS1-KSEV, and GST-avNS1-RSKV, respectively. Additionally, a GST-avNS1-ESEA (modified H6N6 A/blue-winged teal/MN/993/1980) and a human NS1 (H3N2 A/Memphis/14/1998) with the RSKV PBM at its C terminus, termed GST-huNS1-RSKV, were used as specificity controls.

Epitope-tagged PDZ protein expression plasmids were transfected into 293T cells, and cell extracts were subjected to pulldown assays with GST-NS1 ED proteins immobilized on glutathione-Sepharose beads. The binding reaction products were examined in immunoblot assays (Fig. 2A); Coomassie blue stains showed equivalent levels of GST-NS1 ED proteins were used in the binding assays (data not shown). As observed previously, neither MUPP1 nor PATJ associated with any of the GST-NS1 proteins. Surprisingly, only an NS1 ED with the ESEV PBM associated with Scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3. These data indicate that the EPEV, KSEV, and RSKV PBMs in the context of an H6N6 NS1 ED are unable to bind these PDZ proteins. As expected, the H3N2 NS1 ED with the RSKV PBM did not associate with any of these proteins, as reported previously (32).

To further characterize the NS1 PBM, we examined two NS1 proteins from highly pathogenic H5N1 viruses with a canonical ESEV PBM and a noncanonical EPEV PBM. A previous mouse infection study found that recombinant influenza virus that expressed NS1 proteins with ESEV or EPEV PBMs exhibited increased morbidity and mortality compared to PBM-truncated NS1 proteins (23). We constructed GST-NS1 ED fusion proteins from the H5N1 NS1 proteins termed GST-H5N1NS1-ESEV (H5N1 A/Viet Nam/1194/2004) and GST-H5N1NS1-EPEV (H5N1 A/Hong Kong/156/97). Additionally, a GST-H5N1NS1-ESEA (H5N1 A/Viet Nam/1194/2004) and a GST-H5N1NS1-EPEA (H5N1 A/Hong Kong/156/97) were constructed to serve as specificity controls. Binding assays were performed as described above for the H6N6 GST-avNS1 ED proteins. As seen in Fig. 2B, Scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3 associated with the GST-H5N1NS1-ESEV ED protein but not with the GST-

H5N1NS1-EPEA, -ESEA, or -EPEV ED protein (EPEA and ESEA not shown). These data suggest the noncanonical PBM EPEV may target a different subset of PDZ proteins or even non-PDZ proteins.

The GST-NS1 ED *in vitro* binding assays do not address possible contributions of the NS1 RBD on the PBM's ability to associate with cellular targets. To investigate the binding properties of PBMs in the context of a full-length NS1 protein, we constructed expression plasmids for the H6N6 avNS1 protein with EPEV, KSEV and RSKV PBM sequences. These H6N6 NS1 proteins were termed avNS1-EPEV, avNS1-KSEV, and avNS1-RSKV. FLAG-tagged expression plasmids for the full-length human NS1 with the RSKV PBM, termed huNS1-RSKV, and the avNS1-ESEA proteins served as specificity controls. Additionally, we constructed two full-length FLAG-tagged H5N1 NS1 proteins with the ESEV and EPEV PBM, termed H5N1NS1-ESEV (H5N1 A/Viet Nam/1194/2004) and H5N1NS1-EPEV (H5N1 A/Hong Kong/156/97). Following transfection of these expression plasmids into 293T cells, cell extracts were coimmunoprecipitated with anti-FLAG antibodies, and precipitated proteins were visualized by immunoblotting (Fig. 2C). The p85 β subunit of phosphatidylinositol 3-kinase (PI3K) coimmunoprecipitated with all NS1 proteins as expected (data not shown), as its NS1 binding site lies within ED (44). Consistent with the GST-NS1 ED *in vitro* binding assays, only the avNS1-ESEV and H5N1NS1-ESEV coimmunoprecipitated with endogenous Scribble and Dlg1. The avNS1-ESEV and H5N1NS1-ESEV proteins also clearly associated with endogenous Lin7C, presumably in a complex with Dlg1 (see Fig. 1C).

PBM-dependent colocalization of NS1 with Dlg1 and Scribble and disruption of tight junction markers during a viral infection. We previously constructed recombinant H3N2 influenza A viruses (Udorn strain) encoding an H6N6 NS1 protein with either a wild-type ESEV PBM or a mutant ESEA PBM, enabling the functional analyses of the NS1 PBM during influenza A virus infections in cultured mammalian cells. We found that infections with the ESEV but not mutant ESEA virus resulted in the partition of Scribble into perinuclear puncta that colocalized with the NS1-ESEV protein (32). We used these recombinant viruses to examine the localization of Dlg1 and Lin7C during infection. A549 cells were infected with the ESEV or ESEA virus and analyzed by immunofluorescence at 10 h postinfection. Infection with mutant ESEA virus does not alter the localization of Scribble or Dlg1 from the cell membrane, as the localization of these proteins was similar in mock-infected cells (Fig. 3A). In contrast, infection with the ESEV virus resulted in the sequestration of Dlg1 to perinuclear puncta that colocalized with NS1, as seen previously with Scribble. Significant levels of all three proteins colocalized as white puncta in a merged enlargement (Fig. 3B). It should be noted that most NS1 is nuclear at this time point and the majority of NS1 proteins found within the cytoplasmic puncta appear to colocalize with at least one PDZ protein. Previous data have shown that Scribble, Dlg1, and NS1 partition into an insoluble cell fraction when extracts are prepared at 6 h postinfection, suggesting that these cytoplasmic puncta containing the three proteins represent an insoluble cell fraction (32). In the case of Scribble, the relocalization correlated with the loss of Scribble's proapoptotic function and enhanced viral repli-

FIG. 2. *In vitro* binding assays with NS1 PBMs from a variety of viral isolates. (A) 293T cells were transfected with expression plasmids for the indicated epitope-tagged PDZ proteins, cell extracts were prepared at 48 h posttransfection, and binding assays were performed with GST-NS1 EDs containing the indicated PBM sequences. Products of binding reactions were examined in immunoblots. Coomassie blue stains of the products of binding reactions confirmed that equivalent amounts of GST-NS1 fusion proteins were used in binding assays (not shown). (B) H5N1 and H6N6 GST-NS1 ED fusion proteins were used in binding assays with extracts from 293T cells transfected with the PDZ protein expression plasmids indicated in panel A. The products of binding assays were resolved by SDS-PAGE and examined by immunoblotting. Gels were also stained with Coomassie blue to demonstrate that equivalent levels of GST-NS1 ED proteins were used in binding assays (not shown). (C) 293T cells were transfected with full-length H6N6 or H3N2 FLAG-tagged NS1 protein with the indicated PBMs. Cell extracts were prepared 24 h posttransfection and used in coimmunoprecipitations. Products of immunoprecipitations were examined by immunoblotting.

cation (32). The data shown suggest that Dlg1 may also be functionally inactivated as it is sequestered within puncta containing Scribble and NS1 proteins. We also examined Lin7C localization during infection with ESEA and ESEV virus (Fig. 3C). In mock-infected and ESEA virus-infected cells, Lin7C remained intact at the cell membrane, as seen with Scribble and Dlg1. However, in cells infected with the ESEV virus, Lin7C was partially mislocalized away from normal continuous staining at the cell border and relocalized to cytoplasmic puncta. Additionally, the relocalized cytoplasmic Lin7C colocalized with Scribble and Dlg1 in ESEV

virus-infected cells, as indicated by the white puncta in the merged image (Fig. 3D). We also examined TJ integrity following infection with the H3N2 recombinant viruses, as there is evidence to suggest downregulation of TJ proteins during influenza infection (60). Additionally, Dlg1 and Scribble have been shown to play important roles in cellular junction integrity (22, 42, 48). We used MDCK cells for these experiments, as this polarized epithelial cell line forms functional TJs. MDCK cells were infected with the ESEV or ESEA virus and examined by immunofluorescence at 10 h postinfection (Fig. 3E). We examined both AJ

FIG. 3. Avian NS1 with ESEV PBM colocalizes with Scribble and Dlg1 in cytoplasmic puncta while disrupting Lin7C and tight junction markers. (A) A549 cells were infected at an MOI of 3 with the indicated influenza viruses. Cells were fixed at 10 h postinfection for immunofluorescence analysis with Dlg1, Scribble, and NS1 antisera. (B) Enlargement of indicated infected cell. Puncta with Scribble, Dlg1, and NS1 are displayed as white; puncta with only NS1 and Dlg1 are displayed as cyan; puncta with Scribble and Dlg1 are displayed as yellow; puncta with NS1 and Scribble are displayed as violet. (C) A549 cells were infected at an MOI of 3 with the indicated influenza viruses, and cells were fixed at 10 h postinfection for immunofluorescence analysis with Lin7C and nucleoprotein antisera. (D) A549 cells were infected at an MOI of 3 with the indicated influenza virus, and cells were fixed at 10 h postinfection for immunofluorescence analysis with Lin7C, Scribble, and Dlg1 antisera. (E) MDCK cells were infected at an MOI of 3 with the indicated viruses. Cells were fixed at 10 h postinfection for immunofluorescence analysis with nucleoprotein, ZO-1, or Occludin antisera.

and TJ markers, as Lin7C and Dlg1 have been shown to associate in complexes at the AJ and to influence TJ proteins (5, 47). We found that infection with ESEV or ESEA virus does not discernibly affect the localization of AJ markers E-cadherin and β -catenin or the TJ markers claudin (data not shown) and ZO-3 (Fig. 3E). However, while uninfected cells and cells infected with the ESEA virus showed the expected TJ localization of ZO-1 and Occludin, cells infected with the ESEV virus instead exhibited increased cytoplasmic staining and discontinuous plasma membrane staining for ZO-1 and Occludin. It is notable that ZO-1 is reported to associate with

Scribble and to be crucial for proper Scribble localization in cells (22). These findings suggest that binding of NS1 to Scribble and Dlg1 may structurally disrupt the TJ in cells.

NS1 ESEV PBM functionally disrupts tight junctions. The disrupted TJ staining of ZO-1 and Occludin in MDCK monolayers infected with the ESEV virus observed in Fig. 3E suggests that the ESEV PBM may allow NS1 to compromise TJ integrity. We therefore examined functional TJ integrity in polarized MDCK monolayers by measuring TER and the apical-basolateral diffusion of inulin. Polarized MDCK monolayers were infected apically with ESEV or ESEA virus, and TER measurements were made at 3 h and 6 h postinfection (Fig. 4A). Mock and ESEA virus-infected monolayers exhibited constant TER at both time points, despite ESEA and ESEV having similar numbers of infected cells. Monolayers were processed for immunofluorescence at 6 h postinfection and scored for the presence of NP; ESEA and ESEV monolayers were 35.8% and 36.1% positive for NP, respectively. In contrast, the ESEV-infected monolayers displayed a 10% decrease in TER at 3 h postinfection and a statistically significant 20% decrease at 6 h postinfection. In addition, we observed that MDCK cells depleted of either Dlg1 or Scribble showed significantly reduced TER compared to that in control cells, despite the apparent absence of recognizable TJ structural defects (Fig. 4B and C), suggesting a Dlg1 and Scribble requirement for the barrier function but not overall structural integrity of the TJ (22, 48). Thus, ESEV virus infection partially phenocopies the TER defects of Scribble or Dlg1 depletion in MDCK cells. The data suggest that ESEV virus disrupts the TJ early during infection and that this effect involves NS1 PBM-mediated mislocalization and functional inactivation of Dlg1 and Scribble.

To directly examine cellular permeability, polarized MDCK monolayers were infected with ESEA and ESEV virus, and after the viral adsorption period in the apical chamber, the inoculum was replaced with medium containing FITC-inulin. Samples were removed from the basolateral chamber at 3 h and 6 h postinfection, and amounts of FITC-inulin were determined by fluorescence spectrometry (Fig. 4D). ESEA virus-infected monolayers exhibited diffusion of FITC-inulin to the basolateral chamber to a degree similar to that seen for mock-infected cells, fully consistent with the constant TER measurements shown in Fig. 4A. In contrast, the ESEV-infected monolayers displayed a statistically significant increase in permeability at 3 h postinfection, with 150% of the value of mock-infected FITC-inulin diffusion. This permeability increased at 6 h postinfection to 270% of mock-infected FITC-inulin diffusion. These data confirm that the NS1 protein with the ESEV PBM functionally disrupts the TJ as measured by increased cell monolayer permeability.

DISCUSSION

In this study, we have shown that an avian influenza A virus NS1 protein with the ESEV PBM, a motif found commonly in highly pathogenic H5N1 viruses, mediates binding to Dlg1 and Lin7C. These two cellular PDZ proteins are found in a complex with MPP7, which is involved in cellular polarity (48). The interaction between NS1 and Lin7C appears to be indirect and dependent upon Dlg1, suggesting that NS1 targets the Crumbs complex by binding to Dlg1 (Fig. 1C). This association between

FIG. 4. ESEV PBM of NS1 disrupts tight junctions and increases leakage of infected monolayers. (A) Polarized MDCK cells grown on transwell filter inserts were infected in the apical chamber with an MOI of 1 with the indicated virus or were mock infected. TER was measured at 0, 3, and 6 h postinfection. Readings are expressed as a percentage of mock-infected monolayer TER. Error bars indicate standard deviations from three independent experiments (*, $P < 0.05$ by Student's t test) with at least four transwell inserts per infection condition. Monolayers were processed for immunofluorescence and stained for influenza virus NP and Scribble (data not shown). Five random fields from two monolayers were then counted for the presence of nucleoprotein. ESEV monolayers were 36.1% infected and ESEA monolayers were 35.8% infected. (B) MDCK cells were reverse transfected with siRNA targeting canine Dlg1, canine Scribble, or negative-control siRNA and grown on transwell filter inserts. TER was measured on days 3 and 4 posttransfection. Readings are expressed as a percentage of negative-control siRNA-treated monolayer TER. Data shown are from one representative experiment that was repeated in triplicate. Error bars indicate standard deviations with four transwell inserts per experimental condition (*, $P < 0.05$ by Student's t test). (C) MDCK inserts from panel C were fixed on day 4 posttransfection for immunofluorescence analysis with Dlg1, Scribble, ZO-1, and Occludin antisera. Scribble is displayed as blue, Dlg1 as red, and ZO-1 or Occludin as green. Images are X-Z representations from a Z-stack. (D) Polarized monolayers were infected with indicated viruses at an MOI of 1. After 1 h, the apical inoculum was replaced with medium containing FITC-inulin (2 mg/ml). Samples were removed from the basolateral chamber at 3 and 6 h postinfection and quantified by fluorescence spectrometry. Paracellular permeability was calculated as a percentage of mock permeability. Error bars indicate standard deviations from three independent experiments (*, $P < 0.05$ by Student's t test) with at least four transwell inserts per infection condition.

the influenza virus ESEV NS1 protein and Dlg1 during a virus infection results in sequestration of Dlg1 in perinuclear puncta (Fig. 3). These puncta presumably represent the insoluble protein aggregates observed in our previous study (32), and they exhibit extensive colocalization with Scribble and NS1 and some colocalization with Lin7C.

Both Scribble and Dlg1 are known to be important for TJ function in polarized human epithelial cells. The siRNA knock-down of Scribble leads to disruption of both function and organization of TJs, while depletion of Dlg1 also results in functionally compromised TJs (22, 26, 42). Dlg1 and Lin7C are normally found at AJs (5), and Dlg1 loss can alter the localization of other polarity proteins involved at the TJ. Although TJs can form without Scribble and Dlg1, they are largely non-functional (Fig. 4B and C), suggesting that these two PDZ proteins are critical for TJ functional activity (22, 48). The importance of Scribble and Dlg1 in TJ function established by previous studies agrees well with our finding here that colocalization of these PDZ proteins with the ESEV NS1 protein in perinuclear puncta correlates with a decrease in TJ function as

measured by TER and increased paracellular flux of FITC-inulin (Fig. 4).

While there are no data to indicate that Lin7C is a common target of other viral proteins, a number of previous studies have shown that Dlg1 is specifically targeted through PBMs at the carboxyl termini of proteins encoded by three transforming viruses: HTLV-1 (14), adenovirus (30), and HPV (41, 54). In the majority of these infections, Dlg1 is thought to be functionally inactivated by either proteasome-mediated proteolysis or mislocalization into insoluble complexes, and this inactivation of Dlg1 is thought to disrupt cellular polarity, which contributes to cellular transformation (24, 61).

While influenza A virus is not a transforming virus, the disruption of Scribble, Dlg1, and Lin7C does disrupt TJ function and presumably cellular polarity, and this is likely to benefit viral replication, dissemination in the host, or spread to other hosts. Human Dlg1 and chicken Dlg1 are 90% identical, suggesting that the NS1 protein is likely to target the avian protein through interactions similar to those characterized in this study for the human Dlg1 protein. Human Lin7C and

chicken Lin7C display even greater conservation, with 99% identity. There may be multiple consequences to the inactivation of these PDZ proteins, as they are involved in multiple pathways within the cell (24, 48). Additionally, two recent studies have reported that the ESEV PBM associates with PDLim2 and PSD-95, and it has been speculated that these NS1 targeting these additional PDZ proteins may dampen the innate immune response (63, 64).

Human infections of highly pathogenic H5N1 influenza A viruses display a range of pathologies. The severity of symptoms can range from moderate symptoms typically seen in seasonal influenza virus infections to severe diarrhea, edema within the lungs, encephalitis, cytokine storm, lymphopenia of the extremities, full organ failure, and death (9, 10, 37, 56). Viral dissemination is commonly extensive in H5N1 infections, as both virions and viral RNA can be detected in a variety of tissues: upper and lower respiratory tracts (37), spleen, intestines (56), blood, and brain (9, 10). It is not understood how this broad dissemination occurs, although it may involve an increase in cytokine expression that increases the permeability of infected tissues (7, 60). A number of viral determinants may be involved in dissemination of H5N1 virus during infection, and the NS1 ESEV PBM may be a new mechanism of virus dissemination. The disruption of TJs at key virus replication sites through the association of the ESEV PBM protein with Dlg1, Scribble, Lin7-C, and other PDZ protein targets could allow the virus to have broad access to multiple tissues and organs within the host. In addition, the disruption of TJs may be further exacerbated by the increase in cytokines found during H5N1 influenza A virus infection.

In conclusion, we have presented evidence here that an NS1 protein with the ESEV PBM specifically associates with Dlg1, Lin7C, and Scribble during infection. The interaction of NS1 with Dlg1 and Scribble presumably functionally inactivates these PDZ proteins by sequestering them within insoluble perinuclear puncta. The association between Lin7C and NS1 appears to be indirect and is dependent upon Dlg1; this association between NS1 and Lin7C results in the mislocalization of Lin7C from AJs. While the AJs appear to remain structurally intact during infection, the TJs are disrupted as determined by the relocalization of ZO-1 and Occludin; this disruption of TJ proteins is accompanied by a loss of TJ function as measured by an increase in monolayer permeability and decreased TER. The disruption of TJ function by the ESEV NS1 protein may contribute to increased pathogenesis of viruses that encode the ESEV PBM, especially the currently circulating H5N1 viruses.

ACKNOWLEDGMENTS

This work was supported by grants 5U54AI057156-05 and R21AI083396 (to A.P.R.) and R01CA058541 (to R.T.J.).

We thank Rita Czako for recombinant Lin7C experiments and the Integrated Microscopy Core and Budi Utama for assistance with deconvolution and confocal microscopy. We also thank Sue Crawford for critical comments on the manuscript and technical assistance with TER experiments.

REFERENCES

1. Bao, Y., P. Bolotov, D. Dernovoy, B. Kiryutin, and T. Tatusova. 2007. FLAN: a web server for influenza virus genome annotation. *Nucleic Acids Res.* **35**:W280–W284.
2. Barton, E. S., et al. 2001. Junction adhesion molecule is a receptor for reovirus. *Cell* **104**:441–451.

3. Bilder, D., M. Li, and N. Perrimon. 2000. Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**:113–116.
4. Bilder, D., and N. Perrimon. 2000. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**:676–680.
5. Bohl, J., N. Brimer, C. Lyons, and S. B. Vande Pol. 2007. The stardust family protein MPP7 forms a tripartite complex with LIN7 and DLG1 that regulates the stability and localization of DLG1 to cell junctions. *J. Biol. Chem.* **282**:9392–9400.
6. Bornholdt, Z. A., and B. V. V. Prasad. 2008. X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus. *Nature* **456**:985–988.
7. Bruewer, M., et al. 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *J. Immunol.* **171**:6164–6172.
8. Coyne, C. B., L. Shen, J. R. Turner, and J. M. Bergelson. 2007. Coxsackievirus entry across epithelial tight junctions requires occludin and the small GTPases Rab34 and Rab5. *Cell Host Microbe* **2**:181–192.
9. de Jong, M. D., et al. 2005. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N. Engl. J. Med.* **352**:686–691.
10. de Jong, M. D., et al. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.* **12**:1203–1207.
11. Evans, M. J., et al. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**:801–805.
12. Frese, K. K., et al. 2006. Oncogenic function for the Dlg1 mammalian homolog of the Drosophila discs-large tumor suppressor. *EMBO J.* **25**:1406–1417.
13. Glaunsinger, B. A., S. S. Lee, M. Thomas, L. Banks, and R. T. Javier. 2000. Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. *Oncogene* **19**:5270–5280.
14. Grassmann, R., M. Aboud, and K. T. Jeang. 2005. Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* **24**:5976–5985.
15. Guttman, J. A., and B. B. Finlay. 2009. Tight junctions as targets of infectious agents. *Biochim. Biophys. Acta* **1788**:832–841.
16. Hale, B. G., R. E. Randall, J. Ortin, and D. Jackson. 2008. The multifunctional NS1 protein of influenza A viruses. *J. Gen. Virol.* **89**:2359–2376.
17. Harris, B. Z., and W. A. Lim. 2001. Mechanism and role of PDZ domains in signaling complex assembly. *J. Cell Sci.* **114**:3219–3231.
18. Herrmann, C. H., and A. P. Rice. 1995. Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. *J. Virol.* **69**:1612–1620.
19. Herrmann, C. H., and M. A. Mancini. 2001. The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. *J. Cell Sci.* **114**:1491–1503.
20. Herrmann, C. H., R. G. Carroll, P. Wei, K. A. Jones, and A. P. Rice. 1998. Tat-associated kinase, TAK, activity is regulated by distinct mechanisms in peripheral blood lymphocytes and promonocytic cell lines. *J. Virol.* **72**:9881–9888.
21. Hirata, A., et al. 2004. PDZ domain-binding motif of human T-cell leukemia virus type 1 Tax oncoprotein augments the transforming activity in a rat fibroblast cell line. *Virology* **318**:327–336.
22. Ivanov, A. I., et al. 2010. Tumor suppressor scribble regulates assembly of tight junctions in the intestinal epithelium. *Am. J. Pathol.* **176**:134–145.
23. Jackson, D., M. J. Hossain, D. Hickman, D. R. Perez, and R. A. Lamb. 2008. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. *Proc. Natl. Acad. Sci. U. S. A.* **105**:4381–4386.
24. Javier, R. T. 2008. Cell polarity proteins: common targets for tumorigenic human viruses. *Oncogene* **27**:7031–7046.
25. Keith, D., and A. El-Husseini. 2008. Excitation control: balancing PSD-95 function at the synapse. *Front. Mol. Neurosci.* **1**:4.
26. Laprise, P., A. Viel, and N. Rivard. 2004. Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. *J. Biol. Chem.* **279**:10157–10166.
27. Latorre, I. J., et al. 2005. Viral oncoprotein-induced mislocalization of select PDZ proteins disrupts tight junctions and causes polarity defects in epithelial cells. *J. Cell Sci.* **118**:4283–4293.
28. Lee, H. J., and J. J. Zheng. 2010. PDZ domains and their binding partners: structure, specificity, and modification. *Cell Commun. Signal.* **8**:8.
29. Lee, S. S., B. Glaunsinger, F. Mantovani, L. Banks, and R. T. Javier. 2000. Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. *J. Virol.* **74**:9680–9693.
30. Lee, S. S., R. S. Weiss, and R. T. Javier. 1997. Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. *Proc. Natl. Acad. Sci. U. S. A.* **94**:6670–6675.
31. Leonoudakis, D., L. R. Conti, C. M. Radeke, L. M. M. McGuire, and C. A. Vandenberg. 2004. A multiprotein trafficking complex composed of CASK, Veli, and Mint1 is associated with inward rectifier Kir2 potassium channels. *J. Biol. Chem.* **279**:19051–19063.
32. Liu, H., et al. 2010. The ESEV PDZ-binding motif of the avian influenza A

- virus NS1 protein protects infected cells from apoptosis through directly targeting Scribble. *J. Virol.* **84**:11164–11174.
33. Massimi, P., N. Gammoh, M. Thomas, and L. Banks. 2004. HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene* **23**: 8033–8039.
 34. Nakagawa, S., and J. M. Huibregtse. 2000. Human Scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol. Cell. Biol.* **20**:8244–8253.
 35. Nava, P., S. López, C. F. Arias, S. Islas, and L. González-Mariscal. 2004. The rotavirus surface protein VP8 modulates the gate and fence function of tight junctions in epithelial cells. *J. Cell Sci.* **117**:5509–5519.
 36. Nazli, A., et al. 2010. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog.* **6**:e1000852.
 37. Nicholls, J. M., et al. 2007. Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. *Nat. Med.* **13**:147–149.
 38. Noah, D., K. Y. Twu, and R. M. Krug. 2003. Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAs. *Virology* **307**:386–395.
 39. Nourry, C., S. G. N. Grant, and J. P. Borg. 2003. PDZ domain proteins: plug and play! *Sci. STKE* **2003**:RE7.
 40. Obenauer, J. C., et al. 2006. Large-scale sequence analysis of avian influenza isolates. *Science* **311**:1576–1580.
 41. Pim, D., V. Tomaic, and L. Banks. 2009. The human papillomavirus (HPV) E6⁺ proteins from high-risk, mucosal HPVs can direct degradation of cellular proteins in the absence of full-length E6 protein. *J. Virol.* **83**:9863–9874.
 42. Qin, Y., C. Capaldo, B. M. Gumbiner, and I. G. Macara. 2005. The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *J. Cell Biol.* **171**:1061–1071.
 43. Sajjan, U., Q. Wang, Y. Zhao, D. C. Gruenert, and M. B. Hershenov. 2008. Rhinovirus disrupts the barrier function of polarized airway epithelial cells. *Am. J. Respir. Crit. Care Med.* **178**:1271–1281.
 44. Shin, Y. K., Q. Liu, S. K. Tikoo, L. A. Babiuk, and Y. Zhou. 2007. Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. *J. Gen. Virol.* **88**:13–18.
 45. Songyang, Z., et al. 1997. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* **275**:73–77.
 46. Soubies, S. M., et al. 2010. Species-specific contribution of the four C-terminal amino acids of influenza A virus NS1 protein to virulence. *J. Virol.* **84**:6733–6747.
 47. Straight, S. W., L. Chen, D. Karnak, and B. Margolis. 2001. Interaction with mLin-7 alters the targeting of endocytosed transmembrane proteins in mammalian epithelial cells. *Mol. Biol. Cell* **12**:1329–1340.
 48. Stucke, V. M., E. Timmerman, J. Vandekerckhove, K. Gevaert, and A. Hall. 2007. The MAGUK protein MPP7 binds to the polarity protein hDlg1 and facilitates epithelial tight junction formation. *Mol. Biol. Cell* **18**:1744–1755.
 49. Suzuki, T., Y. Ohsugi, M. Uchida-Toita, T. Akiyama, and M. Yoshida. 1999. Tax oncoprotein of HTLV-1 binds to the human homologue of Drosophila discs large tumor suppressor protein, hDLG, and perturbs its function in cell growth control. *Oncogene* **18**:5967–5972.
 50. Tafazoli, F., C. Q. Zeng, M. K. Estes, K. E. Magnusson, and L. Svensson. 2001. NSP4 enterotoxin of rotavirus induces paracellular leakage in polarized epithelial cells. *J. Virol.* **75**:1540–1546.
 51. Takizawa, S., et al. 2006. Human scribble, a novel tumor suppressor identified as a target of high-risk HPV E6 for ubiquitin-mediated degradation, interacts with adenomatous polyposis coli. *Genes Cells* **11**:453–464.
 52. Thomas, M., C. Kranjec, K. Nagasaka, G. Matlashewski, and L. Banks. 2011. Analysis of the PDZ binding specificities of influenza A virus NS1 proteins. *Virol. J.* **8**:25.
 53. Thomas, M., et al. 2002. Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation. *Oncogene* **21**:5088–5096.
 54. Thomas, M., P. Massimi, C. Navarro, J.-P. Borg, and L. Banks. 2005. The hScrib/Dlg apico-basal control complex is differentially targeted by HPV-16 and HPV-18 E6 proteins. *Oncogene* **24**:6222–6230.
 55. Tonikian, R., et al. 2008. A specificity map for the PDZ domain family. *PLoS Biol.* **6**:e239.
 56. Uprasertkul, M., et al. 2005. Influenza A H5N1 replication sites in humans. *Emerg. Infect. Dis.* **11**:1036–1041.
 57. van Meer, G., and K. Simons. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **5**:1455–1464.
 58. Walters, R. W., et al. 2002. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell* **110**:789–799.
 59. Wang, C. K., L. Pan, J. Chen, and M. Zhang. 2010. Extensions of PDZ domains as important structural and functional elements. *Protein Cell* **1**:737–751.
 60. Wang, S., et al. 2010. Influenza virus-cytokine-protease cycle in the pathogenesis of vascular hyperpermeability in severe influenza. *J. Infect. Dis.* **202**:991–1001.
 61. Wodarz, A., and I. Näthke. 2007. Cell polarity in development and cancer. *Nat. Cell Biol.* **9**:1016–1024.
 62. Yeo, N. K., and Y. J. Jang. 2010. Rhinovirus infection-induced alteration of tight junction and adherens junction components in human nasal epithelial cells. *Laryngoscope* **120**:346–352.
 63. Yu, J., et al. 2011. PDlim2 selectively interacts with the PDZ binding motif of highly pathogenic avian H5N1 influenza A virus NS1. *PLoS One* **6**:e19511.
 64. Zhang, H., et al. 2011. The distinct binding properties between avian/human influenza A virus NS1 and postsynaptic density protein-95 (PSD-95), and inhibition of nitric oxide production. *Virol. J.* **8**:298.
 65. Zielecki, F., et al. 2010. Virulence determinants of avian H5N1 influenza A virus in mammalian and avian hosts: role of the C-terminal ESEV motif in the viral NS1 protein. *J. Virol.* **84**:10708–10718.