BRIEF CONCLUSIVE REPORT

PDZ proteins are expressed and regulated in antigen-presenting cells and are targets of influenza A virus

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Abstract
In this work, we identified the expression, regulation, and viral targeting of Scribble and Dlg1 in antigen-presenting cells. Scribble and Dlg1 belong to the family of PDZ (postsynaptic density (PSD95), disc large (Dlg), and zonula occludens (ZO-1)) proteins involved in cell polarity. The relevance of PDZ proteins in cellular functions is reinforced by the fact that many viruses interfere with host PDZ-dependent interactions affecting cellular mechanisms thus favoring viral replication. The functions of Scribble and Dlg have been widely studied in polarized cells such as epithelial and neuron cells. However, within the cells of the immune system, their functions have been described only in T and B lymphocytes. Here we demonstrated that Scribble and Dlg1 are differentially expressed during antigen-presenting cell differentiation and dendritic cell maturation. While both Scribble and Dlg1 seem to participate in distinct dendritic cell functions, both are targeted by the viral protein NS1 of influenza A in a PDZ-dependent manner in dendritic cells. Our findings suggest that these proteins might be involved in the mechanisms of innate immunity and/or antigen processing and presentation that can be hijacked by viral pathogens.

KEYWORDS
dendritic cells, Dlg, immune targets, influenza NS1 protein, polarity proteins, Scribble

1 | INTRODUCTION

PDZ proteins are scaffold proteins highly conserved throughout species and are involved in a variety of cellular processes such as cell-cell junctions, cell polarity, trafficking or cell signaling among others. These proteins often contain multiple PDZ domains defined as protein interaction modules of approximately 90 amino acids with characteristic secondary structure, originally described in postsynaptic density (PSD95), Disc large (Dlg), and Zonula occludens (ZO-1) proteins, hence the acronym PDZ. The PDZ domain usually binds in sequence-specific manner to short C-terminal peptides, the so-called PDZ-binding motif (PDZbm).

In mammals, the functions of PDZ proteins have been widely studied in polarized cells such as epithelial, where three polarity complexes Par3/Par6/aPKC, Crumbs/Pals/Patj, and Scribble/Dlg/Lgl are well recognized. A network of PDZ proteins involved in epithelial cell polarity is also expressed in lymphocytes. The role of some PDZ polarity proteins, principally those of the Scribble complex, is being documented in T and B lymphocytes. Scribble is indispensable for uropod formation, for the maintenance of the immunological synapse (IS) and, through its interaction with a Class-I MHC-restricted T cell associated molecule (CRTAM), for a phase of attenuated proliferation and cytokine production in activated T lymphocytes. On the other hand, Dlg is involved in T cell signaling and activation as well as in...
CD8+ T cell degranulation during cytotoxicity by granzyme B containing granules. In addition, Dlg has a role in the generation of memory T cells, whereas in B cells Dlg is required to stabilize IgG receptor signaling upon stimulation, hence enhancing memory responses. Additionally, there is compelling evidence that some PDZ proteins might be involved in asymmetric cell division in T cells. However, the role of PDZ proteins in other cells of the immune system remains barely addressed.

Many viruses encode proteins with PDZbm that mimic endogenous ligands and are able to target host PDZ proteins, thus interfering with host PDZ-dependent interactions and affecting cellular mechanisms that in turn favor virus replication. Dlg1 was the first PDZ protein described as a target of 3 viral oncoproteins: the high-risk human papillomavirus E6, human T cell leukemia virus 1 (HTLV-1) Tax, and human adenovirus (Ad) E4-ORF1. To date, many other PDZ proteins, principally those involved in polarity functions, have been recognized as viral targets, and the spectrum of viral proteins that target them is broadening to include nononcogenic proteins. The viral PDZbm interaction with host PDZ proteins may induce their proteasome degradation or their relocation to aberrant subcellular localizations; whichever the mechanism, the endogenous PDZ interaction is disrupted and concomitantly its function.

The influenza A virus (IAV) nonstructural protein NS1 is a key virulence factor for the establishment of the infection. Within its multiple functions, NS1 counteracts the host immune response principally by inhibiting type 1 interferon (IFN-I) functions. NS1 contains a PDZbm that has been related to pathogenicity; the more virulent PDZbm ESEV (glu-ser-glu-val) is mostly present in avian viruses, whereas the less virulent PDZbm RSKV (arg-ser-lys-val) and RSEV (arg-ser-glu-val) are mostly present in human viruses. The ESEV sequence has been shown to interact with the PDZ proteins Dlg1, Scribble, MAGI1, MAGI2, and MAGI3 in vitro, and the targeting of Scribble by NS1 may delay apoptosis in infected epithelial cells.

In the present work, we demonstrated that APCs, monocytes (Mo), macrophages (Mφ), and dendritic cells (DC) express Scribble and Dlg1. These proteins are differentially regulated under cell stimulation conditions and, while both proteins seem to participate in different DC pathways, both are targeted by the IAV NS1 virulence factor in DC.

2 MATERIAL AND METHODS

Detailed material and methods can be found as supplemental material.

2.1 Plasmids and constructs

Plasmid PCAGGS_NS1 was kindly provided by Prof. A. García-Sastre (Department of Microbiology and Global Health and Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, USA). NS1 ORF from A/PR/8/34 IAV was amplified by PCR and subcloned into pCMV-Myc. This plasmid (pCMVMyc_NS1ESEV) bearing the RSEV PDZbm of human viruses was used as template for mutagenesis reactions. Two different PDZbms were generated: the avian virus sequence ESEV (NS1ESEV) and the other human virus sequence RSKV (NS1RSKV). We also eliminated the PDZbm by introducing a STOP codon instead of the amino acid 227 (NS1STOP). A QuikChange II site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA, USA) was used to perform mutagenesis reactions according to the manufacturer’s protocol.

2.2 PCR assays

The expression of Scribble and Dlg1 was analyzed by RT-PCR in Mo, Mφ, immature dendritic cells (iDC), and mature dendritic cells (mDC). Changes in the relative expression of both genes during differentiation and maturation were analyzed by RT-qPCR assays.

2.3 Mφ and DC differentiation and transfections

Peripheral blood mononuclear cells (PBMC) were obtained from human healthy donors. Mo were purified by positive selection with CD14+ magnetic beads using the MACS system (Miltenyi Biotec Inc, Auburn, CA, USA). Mφ and DC were differentiated using standard protocols. For the expression of the different versions of IAV NS1, iDC were transfected with the nucleofector (MAXAMA, LONZA, Basel, Switzerland) according to the manufacturer’s instructions.

2.4 Antigen presentation

Autologous CD4+ T cells specific for Mycobacterium tuberculosis (Mt) H37Ra were generated. These T cells were cocultured with DC from the same individuals pulsed with Mt H37Ra, and conjugates were fixed and stained for immunofluorescence (IF) and confocal analysis.

2.5 Flow cytometry

DC were stained for flow cytometry (FC) analysis 24 h posttransfection. Cells were fixed, permeabilized, and blocked with normal rabbit serum. Anti-Myc tag was used as a primary antibody, and secondary antibody was an anti-mouse Alexa 647. Stained cells were acquired in a FACSARia flow cytometer. Data were analyzed with FlowJo® software.

2.6 SDS-PAGE and Western blot

Cells were resuspended in a lysis buffer and cell lysates quantified for total protein. Equal amounts of sample were subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose, and the expression of each protein was determined with specific antibodies followed by an HRP-linked secondary antibody and Immobilon Western HRP substrate detection kit (Millipore, Billerica, MA, USA).

2.7 IF and confocal microscopy

Cells attached to coverslips were fixed with cold methanol, washed, and blocked. After incubation with primary and secondary antibodies, cells were washed and the coverslips were mounted with ProLong Gold Antifade Mountant (Invitrogen, Life Technologies, Carlsbad, CA, USA) on glass slides. Images were acquired with an Olympus Fluoview FV-1000 laser-scanning confocal microscope. For IF on transfected cells, this assay was performed 24 h posttransfection. Images were quantified using ImageJ software (NIH, Bethesda, MD, USA).
FIGURE 1  Human APCs express PDZ proteins. (A) Specific primers for human Scribble and Dlg1 were assayed by RT-PCR in mRNA from Mo, Mϕ, iDC, and mDC; One representative experiment of at least five is shown. (B) Cell lysates obtained from Mo, Mϕ, iDC, and mDC were analyzed by SDS-PAGE and WB for the expression of Scribble and Dlg1. (C) Densitometric quantification of the proteins normalized to GAPDH is shown; mean ± SD of five independent experiments is shown. (D) Relative expression of scribble and dlg1 was analyzed by qPCR. The mean ± SD of the fold change expression versus Mo is represented for each gene (n = 6); *P < 0.05

3 | RESULTS AND DISCUSSION

3.1 | Expression and regulation of PDZ proteins in human APCs

PDZ proteins are evolutionarily conserved scaffolds that assemble protein complexes involved in many cellular functions. Scribble and Dlg belong to the conserved PDZ proteins involved in polarity regulation. In leukocytes, polarity cues are highly dynamic; it has been documented that both Scribble and Dlg1 participate in the orchestration of many of these transient polarities in T and B lymphocytes. We investigated the expression of the PDZ polarity proteins Scribble and Dlg1 in APC. Human Mo were purified and differentiated into Mϕ and iDC. mDC were generated 24–48 h after the addition of a maturation cocktail to iDC. These populations were evaluated by RT-PCR and Western blot (WB) for the expression of Scribble and Dlg1. The mRNA of both genes was detected in the 4 populations analyzed (Fig. 1A). However, the expression of the proteins was clearly observed in Mϕ, iDC, and mDC, but barely detectable in Mo when analyzed by WB (Fig. 1B). Moreover, both proteins increased their expression when Mo were differentiated into Mϕ (P < 0.05) or iDC (P < 0.05) as well as when iDC matured to mDC (P < 0.05) (Fig. 1D). The mean ± SD of densitometric quantification relative to GAPDH or β-tubulin of 5 independent experiments is shown in Fig. 1C, whereas the corresponding quantification of the representative blot is shown below each membrane (Fig. 1B). Analyses of the relative expression of mRNA by qPCR revealed that Scribble increased when Mo were differentiated into iDC or Mϕ (P < 0.05), whereas Dlg1 increment was statistically significant (P < 0.05) only when Mo were differentiated into iDC. However, in contrast to the increment observed in the protein levels, the mRNA of both Scribble and Dlg1 was down-regulated when iDC mature to mDC (P < 0.05) (Fig. 1D). APCs are key components linking innate and adaptive immune responses. The novel expression of Scribble and Dlg1 in APC, as well as their regulation during differentiation and DC maturation, suggest important roles for these proteins in different mechanisms of the innate immune response and/or antigen presentation.

It is known that the functions of PDZ proteins strongly depend on their subcellular localization. We analyzed the subcellular localization of Scribble and Dlg1 in APC by IF. Both proteins are expressed mainly within the cytosol of Mo, although some nuclear expression can be appreciated (Figs. 2A and 2B); Mϕ and DC displayed cytosolic and nuclear expression of both proteins (Figs. 2A and 2B). The expression profile in a cross section (indicated in Fig. 2A) of a representative cell for each condition is shown (Fig. 2C). In the profile, a slight accumulation of Scribble in the plasma membrane of Mϕ and some mDC can be appreciated (Fig. 2C). The morphology of the cells can be observed in the bright field of each panel (Supplemental Fig. 1). Furthermore, both Scribble and Dlg1 exhibited a punctate expression pattern in all the cells analyzed, suggesting that these proteins localize in a kind of vesicles whose identity is part of ongoing experiments.
FIGURE 2  Cytosolic and nuclear expression of Scribble and Dlg1 in APCs. Mo, Mϕ, iDC, and mDC were methanol fixed and analyzed by IF with a-Dlg1 or a-Scrib primary antibodies. In each case, the secondary antibody was coupled to AlexaFluor 488. (A) Confocal images of one representative experiment from at least eight are shown. (B) Quantification of cytosolic and nuclear mean fluorescence intensity from at least 100 cells from each condition is shown (mean ± SD). (C) The profile of a cross section (indicated in A) of a representative cell from each panel is shown.

Scribble and Dlg function in the same pathway regulating apico-basal cell polarity and both proteins colocalize in the basolateral membrane in epithelial cells. However, these proteins also have distinct functions, most of which are cell specific. With this in mind, we analyzed whether Scribble and Dlg1 colocalize in DC. We demonstrated that colocalization of Scribble and Dlg1 was very low in iDC or mDC (Fig. 3A) as indicated by low Pearson’s coefficient (Fig. 3B), which suggests that these proteins might be involved in the regulation of distinct mechanisms during differentiation and DC maturation.

To analyze the relocation of Scribble and Dlg1 in DC during antigen presentation, we took advantage of a previously established model suitable for the study of antigen presentation in human primary cells; briefly, iDC were matured by infection with the nonvirulent laboratory strain of Mycobacterium tuberculosis Mtb H37Ra and used to stimulate autologous CD4+ specific T lymphocytes from individuals that have been shown to be positive to tuberculin skin test. We observed a strong relocation of Scribble toward the immunological synapse (IS) (Figs. 4A upper panel and 4C), whereas Scribble relocation was absent when iDC cultured in the absence of Mtb H37Ra were put in contact with specific CD4+ T lymphocytes (Figs. 4A bottom panel and 4C). Worth to note, we also observed a slight relocation of Scribble toward the plasma membrane in Mtb H37Ra treated DC (Figs. 4B and 4C, DC+Ag) compared with untreated DC (Fig. 4C, T:DC –Ag) (*P < 0.001). All together, these results suggest that Ag maturation of DC induces a slight relocation of Scribble toward the plasma membrane, whereas Ag presentation to T cell induces Scribble relocation to the IS. In addition, Scribble was located to the distal pole in T lymphocytes after 15–30 min of Ag stimulation (Fig. 4 upper panel), as previously described. On the other hand, we found that the localization pattern of Dlg1 in DC during Ag-presentation remains unaffected (data not shown).

To our knowledge, the unique report regarding a PDZ protein regulation in APC is that of Spinophilin. Spinophilin is a PDZ protein mainly expressed in the central nervous system that localizes to dendritic spines in neurons and to the adherens junction in epithelium. It has been demonstrated that Spinophilin is also expressed in DC;
FIGURE 3  Scribble and Dlg1 might be involved in distinct pathways in DC. The colocalization of Scribble with Dlg1 was evaluated by IF. (A) iDC and mDC were fixed and double-stained with goat α-Scribble and rabbit α-Dlg1. Secondary antibodies were α-goat AlexaFluor 488 and α-rabbit AlexaFluor 647; confocal images from one representative experiment of at least five are shown. (B) The low value of Pearson’s coefficient analysis support the scant colocalization of Scribble and Dlg1 both, in iDC and mDC; no statistical difference was found.

FIGURE 4  Scribble relocates to the IS during antigen presentation. (A) DC cultured in the presence (A upper panel and C, T: DC + Ag) or absence (A lower panel and C, T: DC – Ag) of Mtb H37Ra were used to stimulate Mtb-specific autologous CD4+ T lymphocytes. 30 min after coculture cell conjugates were fixed and double-stained with α-CD3 and α-Scribble. Confocal images were captured. One representative image from at least 3 experiments is shown. (B) Representative image of a DC Mtb H37Ra treated but not in contact with T cells (DC + Ag). (C) Quantification of Scribble IMF in DC. The external circumference of DCs is represented as $360^\circ$ in which $180^\circ$ correspond to the DC:T cell contact site. The IS was identified by the CD3 relocation to the contact zone. A slight relocation of Scribble toward the plasma membrane in Mtb H37Ra treated DC (DC + Ag) versus untreated DC in contact with T cells (T: DC – Ag), *$P < 0.05$

3.2 PDZ proteins are IAV targets in APC

The relevance of PDZ proteins in fundamental cellular functions is reinforced by the fact that PDZ-dependent host interactions could be disrupted by viral proteins that mimic endogenous ligands, thus favoring viral replication. To investigate the potential targeting of these PDZ proteins by viral proteins in APC, we generated IAV NS1 expression plasmids encoding the avian (ESEV) or human (RSKV and RSEV) PDZbm specificities, or an NS1 without a PDZbm (STOP), fused downstream to a myc tag. The four NS1 mutants, NS1ESEV, NS1RSKV, and NS1STOP, were expressed to a similar extent in DC when analyzed by FC (Supplemental Fig. 2).

We transfected iDC with the NS1 plasmids and analyzed the colocalization of NS1 proteins with endogenous Scribble and Dlg1. Interestingly to note, the more virulent NS1ESEV strongly colocalized with Dlg1 (Figs. 5A and 5C) and Scribble (Figs. 5B and 5D) in large cumulus in the cytosol, compared with the control NS1STOP, and with the other NS1 PDZbm specificities; this is supported by the Pearson’s coefficient analyses (Figs. 5C and 5D); *$P < 0.05$; **$P < 0.001$.

In epithelial cells, the specific PDZ-dependent targeting of Scribble by avian IAV NS1 protein induces its relocation from the cytosol to...
perinuclear region, destabilization of the tight junction, and protection of infected cells from apoptosis, thus enhancing IAV replication.\textsuperscript{21,22} We demonstrated the specific PDZ-dependent colocalization of the ectopically expressed avian NS1 from IAV with Scribble and Dlg1, suggesting that these proteins are IAV targets also in APC. During IAV infection, in addition to the epithelial cells of the respiratory tract, resident DC (principally myeloid) and $\gamma$-DC are also susceptible to IAV infection. IAV infection of human DC severely affects their capacity of antigen presentation and cross-presentation with a pronounced reduction in the IAV-specific cytotoxic CD8+ T cell expansion; such dysfunctions are absent with inactivated IAV.\textsuperscript{32} In this context, the IAV nonstructural protein NS1, which is absent in the viral particle but synthesized once the virus infects and start its replication, is an ideal candidate that might be involved in this DC dysfunction. IAV NS1 is a multifunctional protein with a relevant role counteracting the host immune response, with specific inhibitory effects on DC functions that impact their T cell stimulation capacity.\textsuperscript{33,34} We hypothesize that the PDZ-dependent targeting of Scribble and Dlg1 by avian NS1 would be part of the IAV mechanisms to inhibit APC functions and consequently host immune response. The targeting of PDZ proteins by pathogens was originally described for oncoviruses in epithelial cells, but now it is recognized in other cell types and with nononcogenic viruses also, such as rabies virus in neurons,\textsuperscript{35} or IAV itself in epithelial cells,\textsuperscript{22} and now in APC also. Worth to note, it has been recently reported that the p22 subunit of the NADPH oxidase complex expresses a type II PDZbm that interacts with the fourth PDZ domain of Scribble; by means of this interaction, Scribble plays a fundamental role for the assembly of this complex, and thus for the reactive oxygen species production necessary for the elimination of a variety of pathogens.\textsuperscript{36} Interestingly, the NS5 protein of tick-borne encephalitis virus (TBEV) codifies an internal PDZbm able to target the fourth PDZ domain of Scribble, leading to the inhibition of JAK/STAT signaling, thus IFN responses in epithelial cells.\textsuperscript{37} Considering the capacity of TBEV to infect and inhibit DC,\textsuperscript{38} it is reasonable to think that this virus can hijack PDZ-dependent endogenous interactions in APC to favor its own replication.

We demonstrated that Scribble and Dlg1 are expressed and regulated in APC. Moreover, both proteins were shown to be target of the viral protein NS1 of IAV in DC in a PDZ-dependent manner. Taken together, our results support and expand the notion of the conservation of PDZ proteins and account for the relevance of PDZ proteins...
and their interactions in APC, giving rise to intriguing questions regarding the functions of these proteins in APC, their endogenous partners, the multiprotein complexes in which they are involved and their potential to be targeted by viruses. APC are key players in the orchestration of host immune response and we hypothesize they would be targeted by viral proteins bearing PDZbm as a common mechanism to evade immune response.

AUTHORSHIP
T.S.M., D.B., and K.N.C. designed the experiments and wrote the manuscript. D.B., M.S.G., J.L.F., and K.N.C. performed experimental work; D.B., K.N.C., A.S.A., and T.S.M. analyzed the data; K.B. and T.S.M. provided experimental material; T.S.M. conceived of and supervised the study.

ACKNOWLEDGEMENTS
This work was supported by National Council of Science and Technology (CONACYT), Mexico (grants CB2010-155382, CB2015-250579, Catedra 2095, and IMMUNOCANEI253053). We would like to thank Dr. Claudia Vargas-Dominguez, Dr. Vianney Ortiz-Navarrete, Dr. Oscar Medina-Contreras, Dr. Edgar Sevilla-Reyes, and Dr. José Tapia for helpful discussion and support with reagents; Dr. Luis H Gutiérrez-González for editorial assistance.

DISCLOSURE
The authors declare that they have no conflict of interest relating to this study.

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How to cite this article: Barreda D, Sánchez-Galindo M, López-Flores J, et al. PDZ proteins are expressed and regulated in antigen-presenting cells and are targets of influenza A virus. J Leukoc Biol. 2018;103:731–738. https://doi.org/10.1002/JLB.4AB0517-184R