Virus Adaptation and Treatment

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ORIGINAL RESEARCH

Antibody targeting of TSG101 on influenza-infected cells

This article was published in the following Dove Press journal:
Virus Adaptation and Treatment
8 November 2010
Number of times this article has been viewed

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Abstract: Influenza remains a significant cause of morbidity and mortality worldwide. Although vaccination programs and conventional antiviral therapies can reduce disease burden, increasing resistance to conventional therapies renders much of the population susceptible to infection. The present study focuses on an important host protein target, tumor susceptibility gene 101 (TSG101), which is functionally exploited (hijacked) by certain enveloped viruses to facilitate viral budding and release. We find that influenza viruses depend on TSG101 for progeny virion morphogenesis in infected host cells. Antibody-binding studies revealed that TSG101 is exposed at the surface of influenza-infected cells but remains intracellular in uninfected cells. Using recombinant TSG101 and influenza M1 protein, we demonstrated a direct interaction between these proteins involving the ubiquitin E2 variant domain of TSG101. These findings identify an interaction between TSG101 and M1 protein in infected cells. Furthermore, a monoclonal antibody directed against TSG101 reduced virus yields in cell-based assessment of influenza virus infection, underscoring the potential of the TSG101-M1 interaction as a possible antiviral therapeutic target. The display of TSG101 at the surface of infected cells, combined with evidence that TSG101 antibodies reduce virus yields, suggest that TSG101 plays an essential role in the budding process of influenza virus. Our findings may also suggest potential opportunities for influenza treatment and prevention by using monoclonal antibody therapeutics to interfere with virus replication.

Keywords: influenza, TSG101, infected cells, vaccine

Introduction

Influenza is a major cause of morbidity and mortality in the United States. In a typical year, more than 30,000 deaths in the United States are directly attributable to influenza virus infections.[1] Annual vaccination can prevent influenza infection if the correct circulating strain is adequately predicted; however, vaccine coverage is incomplete, leaving large segments of the population vulnerable to infection. Antiviral drugs for influenza are not effective against all strains, and the emergence of drug-resistant viruses is a growing problem.[2] Therefore, there is a great need for novel, broad-spectrum antiviral therapeutics that are minimally susceptible to the emergence of drug-resistant viruses.

Influenza virus pathogenesis requires the formation of complete virions that bud from the plasma membrane of infected host cells. Budding is a complex process involving both virus and host cell components.[3,4] The matrix proteins of RNA viruses regulate viral assembly and budding from the host cell, and the expression of a matrix protein alone is sufficient for the formation of virus-like particles (VLPs). Such findings

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have been reported for human immunodeficiency virus (HIV; via p6 Gag), Ebola virus (via viral protein [VP] 40), and influenza virus (via M1).[5–8]

The fact that matrix protein expression is sufficient for the formation of VLPs raises a question as to whether potential host protein interactions may assist this process. In the case of HIV and Ebola viruses, the late-domain proteins interact with the host cell protein tumor susceptibility gene (TSG101) to facilitate budding.[9–12] TSG101 is a subunit of the endosomal sorting complex required for *t*ransport-1 (ESCRT-1) and is highly conserved throughout mammalian species.[13] TSG101 normally functions to regulate the transit of internalized proteins from the multivesicular body (MVB) to the proteosome.[14] In this role, TSG101 resides exclusively within the MVB and is not exposed on the cell surface. Upon viral infection, the virus machinery recruits TSG101 to facilitate viral budding and release of progeny virions. A similar "hijacking" of TSG101 has been reported for many enveloped viruses and has perhaps been most thoroughly described in HIV-infected cells.[9,11,12,15,16] In this example, the HIV p6 Gag protein contains a PTAP late-domain motif, which interacts with the ubiquitin E2 variant (UEV) domain of TSG101.[9,11,17] This interaction redirects the function of TSG101 to facilitate viral budding at the plasma membrane.

In our present article, we demonstrate that TSG101 regulates the release of influenza viral particles from infected cells. Although TSG101 is normally restricted to the cytoplasm, it is exposed on the outer surface of influenza-infected cells. We further show that the UEV domain of TSG101 interacts with influenza matrix protein, M1. Finally, we demonstrate that the exposure of TSG101 on the surface of infected cells offers an opportunity for therapeutic intervention using monoclonal antibodies.

Methods

Antibodies

Polyclonal antibody 1299 was generated by immunizing a rabbit against a 19-amino acid peptide derived from the *N*-terminal region of TSG101 (TIKTGKHVDANGKIYLPYL); whereas polyclonal antibody 1529 was generated by the immunization of a rabbit with a 24-amino acid peptide derived from the *C*-terminal region of TSG101 (LRRGVIDLDVFLKHVRLLSTKQFQ). To generate monoclonal antibodies, rabbits were immunized with a 25-kD fragment corresponding to the *N*-terminal portion of TSG101, which includes the UEV domain of TSG101. The 4-8A4 hybridoma and other rabbit monoclonal antibodies were identified by screening the cell culture supernatants with TSG101 using enzyme-linked immunosorbent assay (ELISA) and cell surface staining of influenza-infected cells (see below). All antibodies were purified using protein A column. Rabbit normal immunoglobulin (Epitomics, Burlingame, CA) or isotoype-matched (IgG1) rabbit monoclonal antibodies provided negative controls.

Proteins

The expression vector pET-21b, containing the UEV domain of TSG101, was generously provided by Dr M Javad Aman (United States Army Medical Research Institute for Infectious Diseases). The UEV domain was expressed as a *C*-terminal His-tag fusion protein in the *Escherichia coli* strain BL21 and purified with a nickel column. The *C*-terminus of TSG101 was inserted into the expression vector pLLexp and expressed as a 3′ Fc-fusion protein in the 293 cell lines. The fusion protein was purified with a protein A column. The full-length TSG101 protein was expressed as an *N*-terminal His-tag fusion protein in 293 or HeLa cells by Q-Biogene Inc (Carlsbad, CA). The fusion protein was purified under denaturing conditions and refolded with Pierce's protein refolding kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL) following the manufacturer's instruction. The influenza M1 protein gene from influenza A/NewYork/312/200, graciously provided by Dr Jeff Taubenberger at the National Institutes of Health (NIH, Bethseda, MD), was cloned in the expression vector pET41a, and expressed as a 3′ glutathione S-transferase (GST)-His tag fusion protein in the *E. coli* strain BL21. The M1 protein was released from the fusion protein with thrombin protease after purification with a nickel column.

Cell culture and virus infections

Madin–Darby canine kidney (MDCK) cells were purchased from the American Type Tissue Culture collection (Manassas, VA) and were grown in minimum essential medium (MEM) containing 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C under a 5% CO_2/95% air atmosphere. All influenza virus strains (see Figure 3A) were obtained from Charles River Laboratories (Wilmington, MA) and grown in embryonated eggs, and the titers were determined by plaque assay. MDCK cells were infected by removing the culture medium and adding the appropriate multiplicity of infection (MOI) of virus in infection media consisting of MEM, 0.2% bovine serum albumin (BSA; Sigma-Aldrich®, St Louis, MO), 100 U/mL penicillin, and 100 μg/mL streptomycin and tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. After incubation for 1 hour at 37°C, virus was removed, cells were rinsed with

phosphate-buffered saline (PBS; GIBCO-BRL, San Diego, CA) and cultured at 37°C under 5% CO_2 for the amount of time indicated. For virus inhibition studies, antibody was added 1 hour after virus inoculation. Transfections with the TSG-3′ expression vector pcGNM2/TSG-3′ (kindly provided by E Freed, National Cancer Institute, NIH) were performed using the lipofectamine 2000 kit (Invitrogen, Carlsbad, CA) following the manufacturers' instructions. Expression levels were confirmed by western blot.

Rabbit normal immunoglobulins (Epitomics) were used as isotype control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin was purchased from Becton-Dickinson (San Jose, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin was purchased from GE Healthcare (New York, NY).

Virus titer assays

Virus titers were determined using a standard plaque assay. Briefly, 0.3 mL of nine 10-fold serial dilutions of infected cell culture supernatants were added in duplicate to MDCK cells cultured on 6-well plates. The virus-containing supernatant was allowed to absorb to the MDCK cells for 1 hour at 37°C. Following the 1 hour incubation, the cultures were washed with PBS and overlaid with 1% agarose dissolved in virus infection media described above. Plates were incubated at 37°C in 5% CO_2 for 3 days. At the end of the incubation, cells were fixed for 1 hour using formaldehyde, followed by removal of the agarose overlay. The fixed cells were stained with crystal violet for 30 minutes and washed with water to remove excess stain. Plaques were counted and titers were determined using the following equation:

$$\text{PFU/mL} = (\text{Number of plaques} \times [1/\text{dilution factor}])/0.3$$

Flow cytometry and indirect immunofluorescence assay

For flow cytometry analysis, MDCK cells were suspended using 0.05% trypsin, and 1×10^5 cells were incubated on ice with 10 μg/mL TSG101 antibody for 30 minutes. Cells were washed 3 times with PBS containing 1% BSA (Sigma-Aldrich) on ice and incubated with FITC-conjugated goat anti-rabbit (Becton Dickinson) diluted in PBS-BSA for 30 minutes on ice. Following three washes in wash buffer, the cells were fixed in PBS with 1% paraformaldehyde or used to determine viability. For viability assays, 7-amino-actinomycin D (7AAD; Guava Technologies, Hayward, CA) was added to the unfixed cells according to manufacturer's instructions. Fluorescence data were acquired in an EasyCyte Flow Cytometer (Guava Technologies), and data were analyzed using FlowJo analysis software (Tree Star, Inc, Ashland, OR). For immunofluorescence assay (IFA), MDCK cells were grown on coverslips, rinsed with PBS, and fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 1 hour. For nucleoprotein staining, cells were extracted with 0.1% TritonX-100 (Sigma-Aldrich) for 10 minutes at room temperature. For TSG101 detection, infected cells were rinsed with PBS and stained immediately in the presence of 0.1% sodium azide to suppress endocytosis. The cells were then fixed with 4% paraformaldehyde, incubated with anti-TSG101 antibodies for 30 minutes, washed, labeled with FITC-conjugated goat anti-rabbit antibodies (Becton-Dickinson) for 30 minutes, and then mounted onto slides for microscopic evaluation using a epifluorescence microscope (Nikon, Melville, NY)

Enzyme-linked immunosorbent assay

ELISA plates (ThermoScientific, Waltham, MA) were prepared by the addition of 100 ng of purified influenza A/M1 protein. After an overnight incubation at 4°C, the plates were washed with PBS-Tween and blocked with 2% milk in PBS-Tween for 1 hour. The plates were then washed, and 500 ng of the respective protein (TSG101 full length, UEV or C-term) was added and allowed to bind for 1 hour at room temperature. The plates were then washed 3 times with sample buffer. Bound protein was detected using polyclonal antibody 1299 for the full length and UEV proteins and polyclonal antibody 1529 for the *C*-terminal fragment followed by a goat-anti-rabbit HRP-conjugated antibody.

Results

TSG101 is exposed on the surface of influenza-infected cells

Previous reports demonstrate that the late-domain protein of Ebola virus, VP40, interacts with TSG101 and that this complex relocalizes to the cell membrane.[18] To determine whether influenza virus infection might similarly alter the subcellular localization of TSG101, we generated rabbit polyclonal antibodies to analyze TSG101 distribution in infected cells. MDCK cells were infected with influenza A/Udorn/72 (H3N2) for 18 hours, and TSG101 antibody binding was assessed using fresh (unfixed) samples. Flow cytometric analyses revealed that TSG101 was indeed exposed on the surface of live cells infected with influenza A/Udorn/72, but not on matched, uninfected controls (Figure 1A). TSG101 could be detected in uninfected cells only if they were extracted with detergent before staining (data not shown). TSG101

was detected on the cell surface when multiple antibodies directed towards different regions of the protein were used (antibody 1529 *C*-terminus and antibody 1299 *N*-terminus). Both polyclonal antibodies showed specificity to purified TSG101 in ELISA (Figure 1B). These findings indicate that surface staining of infected cells is not an artifact unique to any particular antibody or epitope. We also confirmed the membrane localization of TSG101 using alternative cell models, with identical findings obtained using influenza-infected laryngeal carcinoma (HEp-2) cells (data not shown).

We considered that viral infection of host cells could compromise the integrity of the cell membrane, thereby allowing TSG101 antibodies to gain access to the cell interior. The vital dye 7AAD is excluded from viable cells and, thus, provided an effective measure of membrane integrity. MDCK cells were infected with influenza for 18 hours before labeling with TSG101 antibodies and 7AAD. The samples were then analyzed using flow cytometry to exclude cells with compromised cell membranes (ie, those with 7AAD uptake). These studies demonstrated that TSG101 was exposed on the surface of the influenza-infected, 7AAD-negative cell population (Figure 2). Similar findings were obtained using Trypan blue exclusion as a means to evaluate membrane integrity (data not shown), thus confirming that TSG101 surface

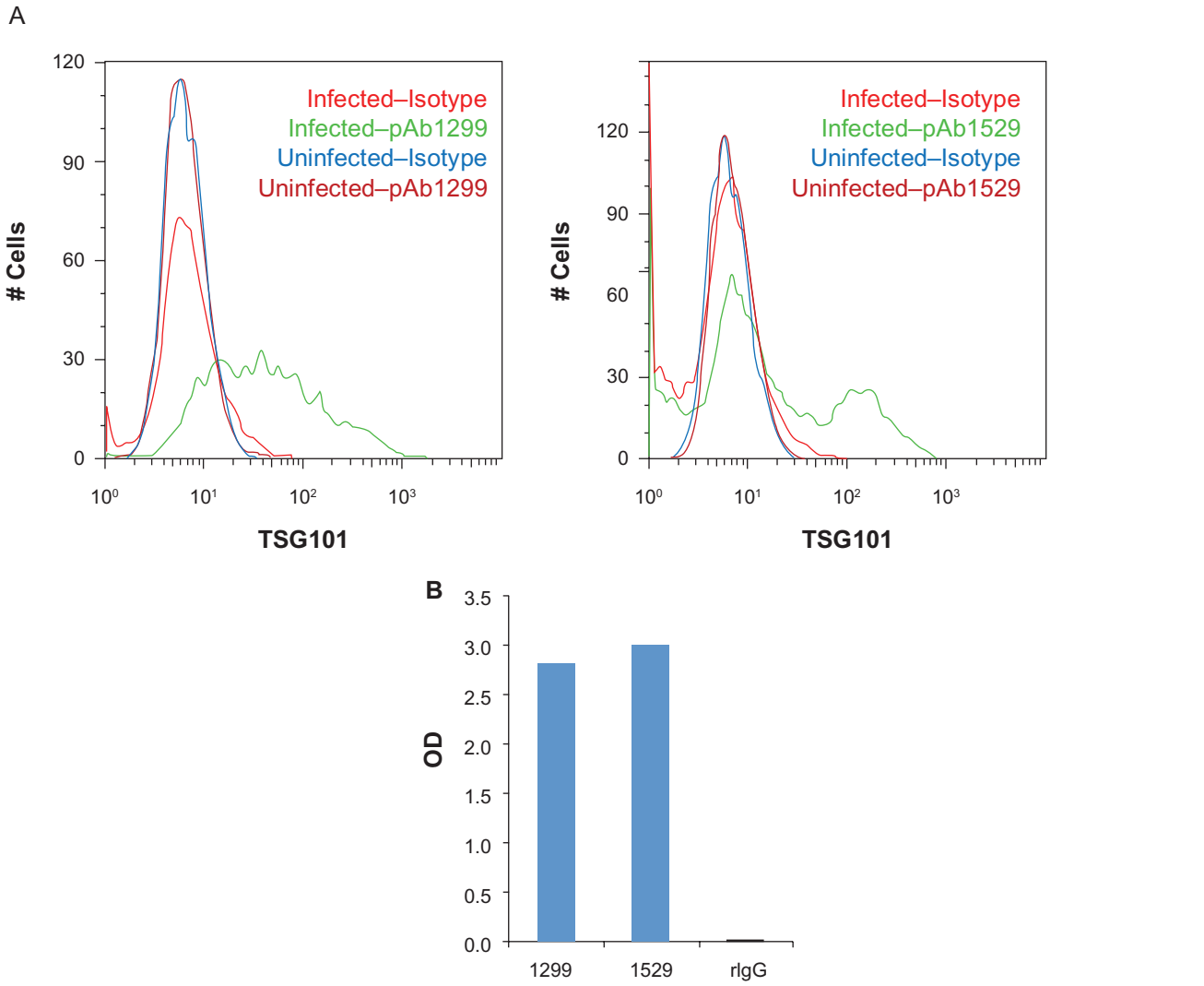


Figure 1 Flow cytometric analysis of TSG101 antibody binding to the surface of influenza-infected cells. MDCK cells were infected with influenza A/Udorn/72 at MOI = 1 for 18 hours. Infected and uninfected MDCK cells were incubated with either rabbit polyclonal antibody 1299 or 1529 rabbit anti-TSG101. FITC-conjugated goat anti-rabbit antibody was used for flow cytometric detection of anti-TSG101 antibody binding. **A**) Histograms comparing TSG101 surface exposure on infected (green) and uninfected cells (brown). Controls include a matched rabbit isotype antibody staining of infected (red) and uninfected (blue) cells. **B**) The results of an ELISA assay used to determine the specificity of antibodies 1299 and 1529 to purified full-length TSG101; a rabbit IgG isotype was used as a negative control. Please note that antibody 1299 recognizes epitopes on TSG101 spanning from residues 96–114 (TIKTGKHVDANGKIYLPYL), whereas antibody 1529 recognizes epitopes that span from amino acid 374 to the *C*-terminal region of TSG101 (QLRALMQKARKTAGLSDL).

Abbreviations: TSG, tumor susceptibility gene 101; rIgG, rabbit immunoglobulin.

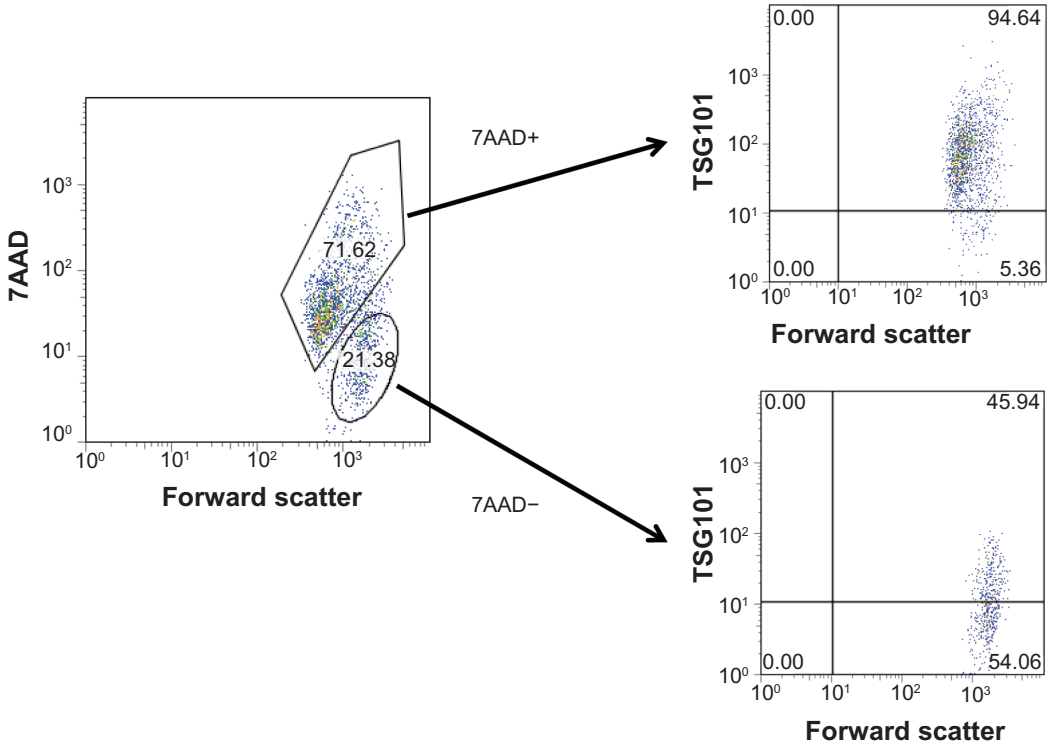


Figure 2 Demonstration of cell membrane integrity on TSG101-positive cells. TSG101 was detected on MDCK cells infected with influenza A/Udorn/72 (MOI = 1) for 18 hours by adding polyclonal antibody 1299 and a FITC-conjugated goat anti-rabbit secondary antibody. Membrane integrity was assessed using 7AAD, which is excluded from viable cells. The dot plot on the left shows the distribution of 7AAD-positive and 7AAD-negative cells in the infected MDCK cultures. The dot plots on the right show TSG101 detection on 7AAD-positive cells and 7AAD-negative cells.

exposure did not result from compromised integrity of the cell membrane.

The studies above focused on influenza A/Udorn/72 (H3N2). Since influenza viruses encompass multiple subtypes, we evaluated other influenza strains. In each case, TSG101 was selectively exposed on the surface of infected cells (Figure 3A). We then asked if TSG101 might behave similarly on emerging avian viruses or previous pandemic strains. For this, the recently circulating strain of avian influenza A/Vietnam/1203/04 (H5N1) or the pandemic strains from 1957 and 1968 were used to infect MDCK cells (see Figure 3B for a representative figure). Similar to our findings with seasonal subtypes, infection with influenza H5N1, H2N2, or H3N2 variants caused TSG101 translocation to the surface of infected cells.

TSG101 cell surface exposure coincides with viral release from infected cells

Given the critical role of TSG101 in the budding of other enveloped viruses, we asked how the timing of TSG101 membrane exposure relates to influenza virus release from infected cells. TSG101 surface exposure on MDCK cells was assessed over time following infection with influenza A/Udorn/72 (Figure 4A). TSG101 was initially detected on the cell surface within 8 hours postinfection with influenza A/Udorn/72 and remained at high levels for the following 24 hours. We then quantified virus release from infected cells and compared this with TSG101 surface staining (Figure 4B). These results indicate that the unique surface exposure of TSG101 coincides with the time course of viral replication or protein production and coincides with virus release.

TSG101 interacts with influenza M1 protein

The UEV domain of TSG101 interacts with the late-domains of certain enveloped virus matrix proteins (eg, HIV p6 Gag or Ebola virus VP40).[12,19] We, therefore, asked if influenza M1 might similarly interact with TSG101. To conduct these assays, influenza M1 protein and different forms of TSG101 were expressed and purified (Figure 5A). An ELISA-based assay was developed by immobilizing M1 protein and assessing the binding of purified TSG101 (Figure 5B). We observed that influenza M1 interacted with TSG101 in a dose-dependent manner. This interaction was specific for TSG101 and was not observed with matched controls (eg, BSA-coated surfaces). We further defined the site of

A

Virus	Subtype	TSG101 surface Immunoreactivity MDCK
Udorn/72	H3N2	+
Hong Kong/8/68	H3N2	+
Puerto Rico/8/34	H1N1	+
Japan/305/57	H2N2	+
Vietnam/1203/04	H5N1	+

B

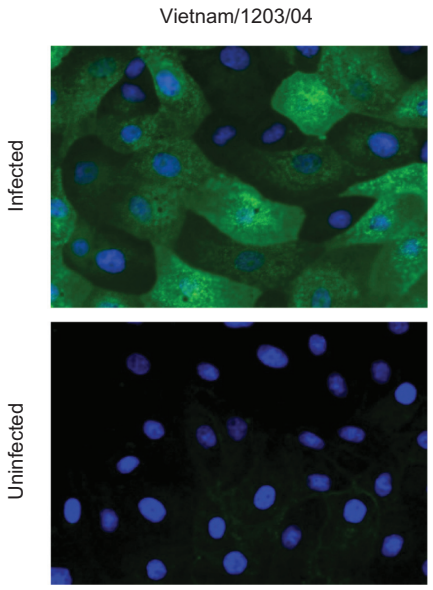


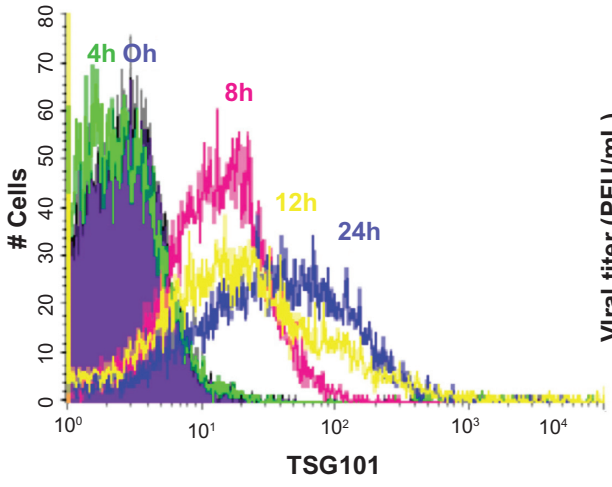
Figure 3 Detection of TSG101 on the surface of cells infected with different strains of influenza A. Cell surface TSG101 was detected using rabbit polyclonal antibody 1299 by immunostaining of MDCK cells infected with **A**) seasonal variants of influenza (MOI = 1). Surface exposure was assessed using flow cytometry (see Figure 1), and a summary of cell surface antibody immunoreactivity is shown. **B**) A similar study was performed following infection of MDCK with the H5N1 avian strain A/Vietnam/1203/04. Labeling of TSG101 was performed by IFA using intact (nonextracted) cells. H5N1-infected cells showed a pattern of fluorescence, characteristic of membrane expression, whereas uninfected cells revealed no specific fluorescence signal.
Abbreviation: MDCK, Madin–Darby canine kidney.

interaction of TSG101 with influenza M1 by performing ELISA assays with two fragments of TSG101 corresponding to the UEV and *C*-terminal regions. The results demonstrate that the UEV region of TSG101 was sufficient to interact with M1 (Figure 5C). Consistent with this finding, the *C*-terminal domain of TSG101 did not interact with influenza M1. These results suggest that M1 interacts directly with TSG101 and that the UEV domain mediates this interaction.

Disruption of TSG101 cellular function inhibits influenza virus release

To elucidate the potential role of TSG101 in the influenza life cycle, we asked whether disruption of TSG101 might impact viral production. TSG-3′ is a truncated form of TSG101 that functions as a dominant negative inhibitor of endogenous TSG101.[20,21] Overexpression of TSG-3′ has been shown to inhibit HIV and murine leukemia virus release from infected

A



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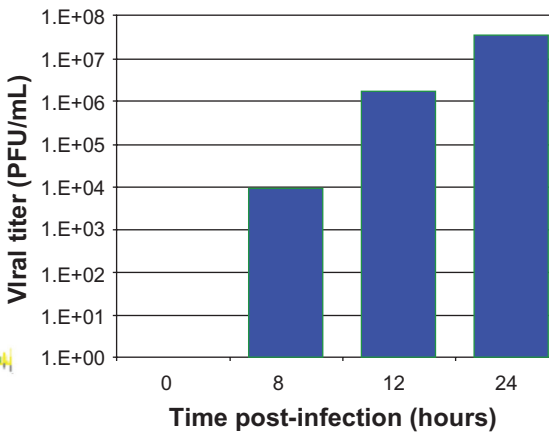

Figure 4 Time course of TSG101 appearance on the surface of influenza-infected cells. MDCK cells were infected with influenza A/Udorn/72 for the times indicated (0–24 hours postinfection), stained with anti-TSG101 (pAb 1299), and evaluated using flow cytometry. **A**) In a parallel study, aliquots of cell supernatants were harvested and plaque assays performed to measure viral output over time (**B**).
Abbreviation: MDCK, Madin–Darby canine kidney.

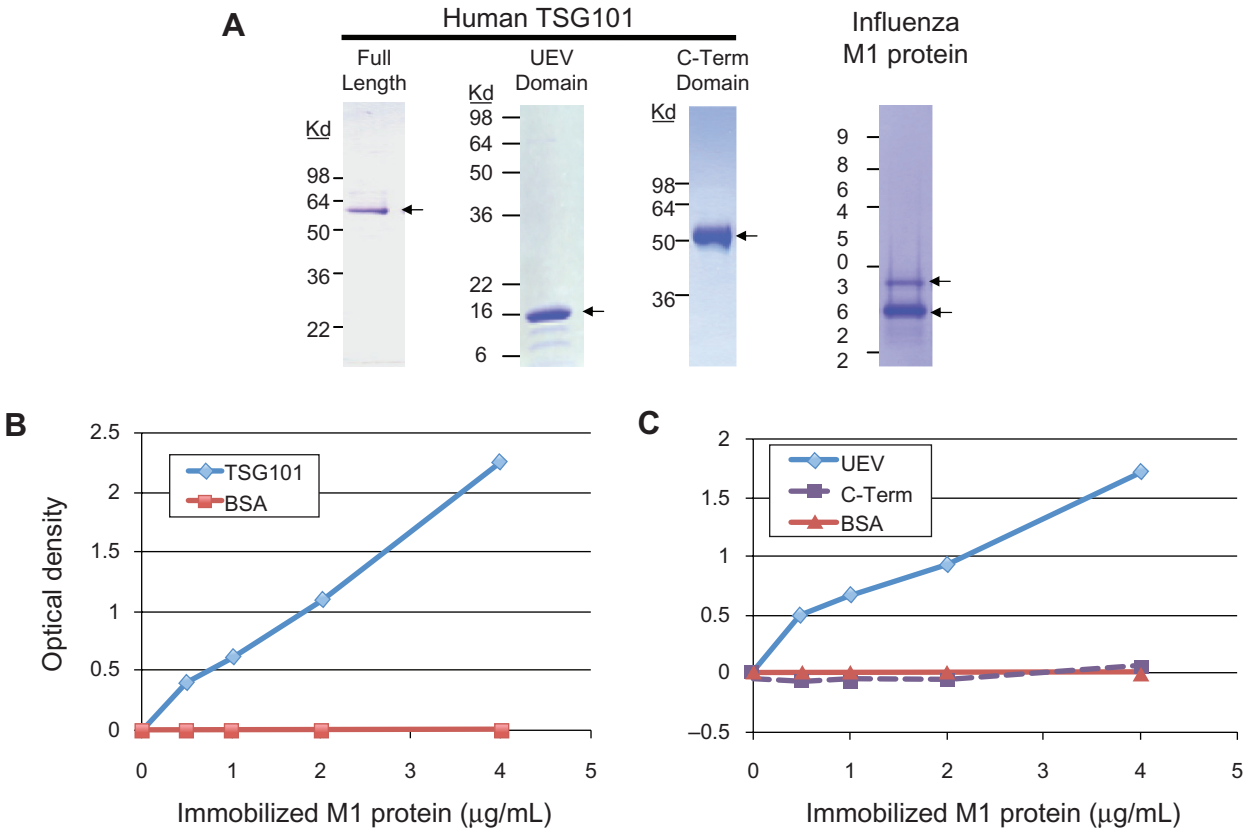

Figure 5 Binding of M1 protein to TSG101. Purified influenza A/New York/312/200 M1 protein was immobilized, and its binding to TSG101 measured using ELISA-based assays. **A**) M1 demonstrates concentration-dependent binding to full-length TSG101 protein, but not to a control protein (BSA). **B**) M1 binding to different portions of TSG101 was assessed, revealing selection recognition of the UEV domain of TSG0101 but not of a fragment corresponding to the *C*-terminal portion of the protein.
Note: The composition of the UEV and *C*-terminal peptides is shown at the bottom of the figure.
Abbreviations: BSA, bovine serum albumin; UEV, ubiquitin E2 variant; BSA, bovine serum albumin.

cells. Transfection of the TSG-3′ construct in MDCK cells decreased virus release by at least 65% when compared with untransfected cells (Figure 6). To evaluate the specificity of this outcome, similar studies were conducted using VPS28, a component of ESCRT-1 that binds TSG101. Transfection with VPS28 did not decrease virus release relative to matched controls. These observations were not unique to a single cell system since similar results were obtained in HEp-2 cells (data not shown).

Antibodies to TSG101 inhibit influenza virus release from infected cells

We then asked if the exposure of TSG101 on the surface of infected cells might provide an opportunity for antibody-based targeted intervention. We developed a rabbit monoclonal antibody, 4-8A4, which recognizes a linear epitope within the UEV domain of TSG101 (residues 35–51). Like the aforementioned polyclonal antibodies, 4-8-A4 specifically recognized the surface of virus-infected cells (Figure 7A). MDCK cells were then infected with influenza A/Udorn/72 at different MOIs in the presence or absence of the TSG101 monoclonal antibody 4-8A4. The release of infectious particles was determined using plaque assays. The inclusion of 4-8A4 decreased the viral titers in a dose-dependent manner (Figure 7B). We then varied the initial MOI. The antiviral effect of 4-8A4 increased with lower initial levels of infection (Figure 7C), which is consistent with the fact that TSG101 plays a role late in the viral life cycle. The results suggest that the appearance of TSG101 on the cell surface upon viral release may be exploited as a therapeutic tool to decrease influenza infection.

Discussion

Many enveloped viruses utilize TSG101 to facilitate budding and release from infected cells.[22] Disruption of the interaction of viral late-domain proteins with TSG101 arrests the budding and release of many different viruses.[9,11,17,23–25] Our present study suggests that TSG101 is similarly "hijacked" in cells infected by influenza A viruses and that this process involves a relocalization of TSG101 from the MVB to the cell surface. We further demonstrate that antibody targeting

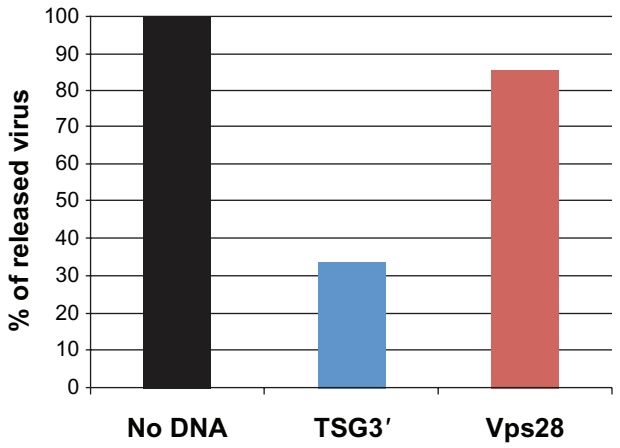

Figure 6 Disruption of TSG101 function inhibits virus release. MDCK cells were transfected with plasmid vectors containing either a dominant negative mutant of TSG101, TSG3′, or VPS28, an ESCRT-I protein known to interact with TSG101. The transfected cells were subsequently infected with influenza A/Udorn/72, and viral output was determined by plaque assay. The graph shows the percentage of virus released from cells transfected with either TSG3′ or VPS28 when compared with the untransfected control.
Abbreviation: MDCK, Madin–Darby canine kidney.

of TSG101 on the surface of infected cells is sufficient to significantly decrease the amount of infectious virus released.

TSG101 localizes with its viral ligand, VP40, to membrane rafts.[18] These prior studies utilized confocal microscopy to show membrane localization but did not address the question of whether TSG101 might be exposed on the outer cell membrane. Our results, thus, provide the first demonstration that TSG101 is exposed on the surface of influenza-infected cells (Figure 1). TSG101 antibodies bound to the surface of live, infected cells, and membrane integrity remained intact based on their ability to exclude dyes that evaluate membrane integrity (Figure 2). Although one interpretation of these data is that TSG101 appears at the surface of infected cells as a direct result of its role in mediating viral budding and release, the mechanistic basis by which TSG101 transits from the internal milieu to the extracellular surface is presently unclear. TSG101 is understood to be one component of a larger ESCRT complex, and other components of this complex (rather than TSG101) may be responsible for this change in subcellular localization. We also cannot exclude that this TSG101 might be released from dead cells and then decorate nearby infected cells. If so, a different explanation would be required to account for the inhibitory activity of certain TSG101 antibodies, and these ideas should serve as subjects for future investigation.

TSG101 antibodies did not bind uninfected cells but appeared on the surface of infected cells during late stages of the viral life cycle (Figure 1). The translocation of TSG101 to the cell surface is concomitant with viral release and is not observed before the appearance of viral particles (Figure 4). We did not observe surface localization of TSG101 in human embryonic lung L-132 cells infected with human coronavirus-229E or simian kidney MA104 cells infected with simian rotavirus SA11–4F (data not shown). Neither of the viruses buds from the cell membrane,[26,27] which is consistent with the absence of TSG101 surface exposure.

Another important outcome of our present study is the idea that TSG101 surface exposure is shared by seasonal and pandemic strains of influenza (Figure 3). The broad usage of TSG101 by divergent influenza viruses is consistent with evidence that TSG101 is "hijacked" by many virus families, including flaviviruses, filoviruses and retroviruses.[9,12,18,22,23,28–38] Such findings suggest a convergent process whereby different virus types have evolved to exploit a similar mechanism. This raises an interesting question for future investigation to ask if TSG101 antibody-based inhibition of influenza might be similarly applicable to these other virus types.

The fact that TSG101 is exclusively exposed on the outer cell membrane of infected cells has interesting implications for the control of influenza infection. Treatment of infected cells with a TSG101 monoclonal antibody reduced the number of infectious viral particles, presumably by blocking viral budding or release (Figure 7). One question that arose during our studies was whether the antiviral effects observed with TSG101 antibodies might arise from interactions with TSG101 on the virus itself. In the case of HIV, truncated forms of TSG101 have been shown to be incorporated into progeny virus.[20] These studies did not elucidate whether full length TSG101 is similarly incorporated into virions or exposed on the viral envelope. Although direct inhibition of TSG101 on the viral surface might be intriguing for future investigation, our present studies do not address this possibility. The evidence presented in this article, however, suggests that the reduction of viral particles as a result of antibody treatment targets specific protein interactions occurring in the infected cell. For instance, the ability to inhibit viral budding was not shared by all TSG101 antibodies and instead relates to the recognition of epitopes within the UEV domain of TSG101.[39] In the case of HIV and Ebola viruses, residues in the UEV domain are necessary for the binding of the late-domain-containing proteins.[9] Consistent with this finding, our data indicate that TSG101 interacts with influenza M1 protein through the UEV domain (Figure 5).

The M1 protein encoded by influenza A viruses contains a putative late-domain motif, YKRL (residues 100–103), and it is conceivable that M1 could facilitate influenza budding similar to the well established interaction of HIV p6

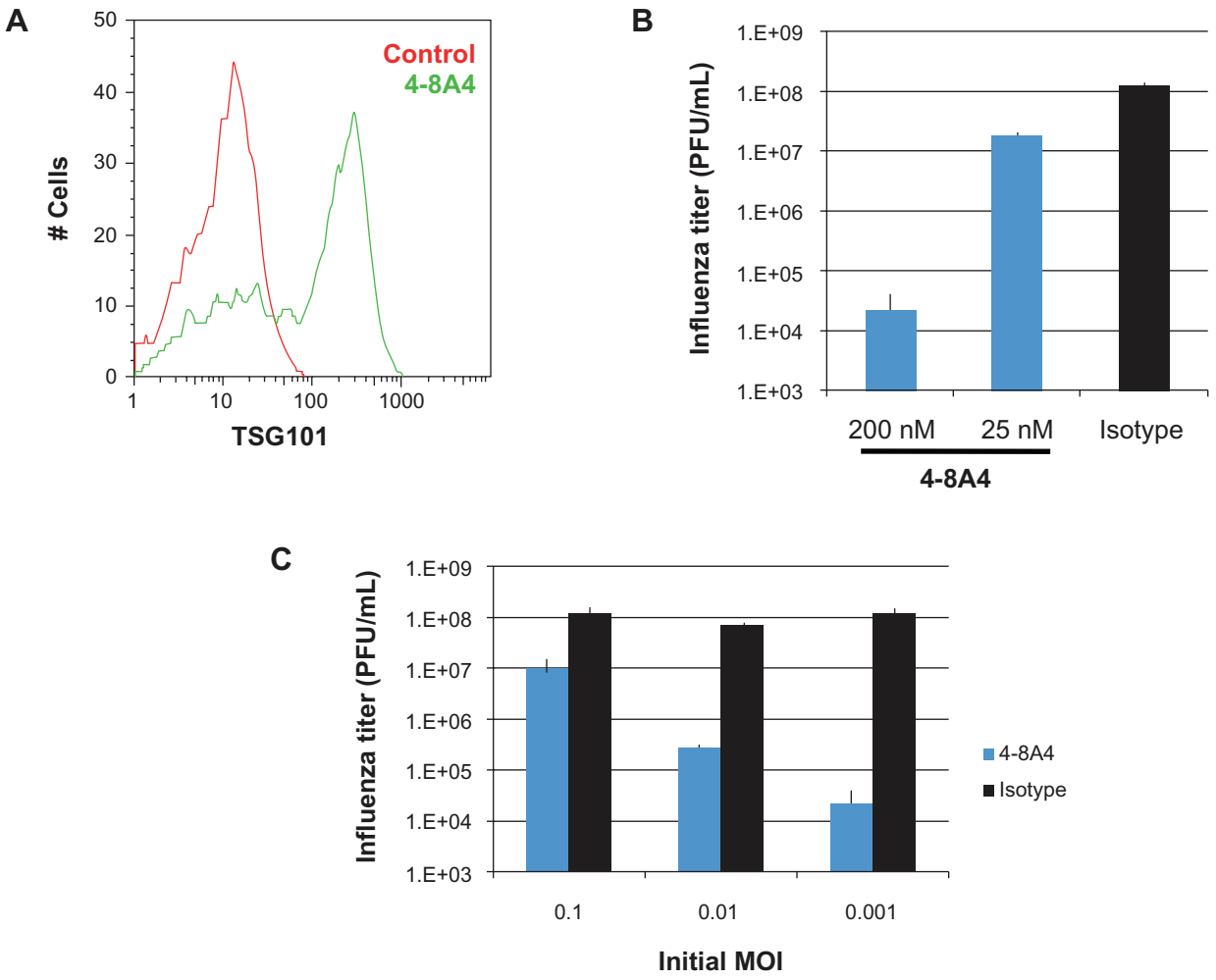

Figure 7 Monoclonal antibody 4-8A4 inhibits influenza virus release. **A**) MDCK cells were infected with influenza A/Udorn/72 (MOI = 1) for 18 hours and then subjected to immunostaining with 4-8A4 and analyzed using flow cytometry. **B**) MDCK cells were infected with influenza A/Udorn/72 (MOI = 0.001) in the presence of the indicated concentrations of TSG101 antibody 4-8A4. Viral titers were assessed in the culture supernatants 24 hours after infection. **C**) MDCK cells were infected with influenza A/Udorn/72, at the indicated MOIs, in the presence (blue bars) or absence (black bars) of TSG101 monoclonal antibody 4-8A4 (200 nM). Twenty-four hours postinfection, supernatants were collected and subjected to plaque assays to determine viral titers.
Abbreviation: MOI, multiplicity of infection.

Gag or Ebola virus-encoded VP40 with TSG101. Further investigation should determine if the YKRL late-domain motif is sufficient to mediate the interaction of M1 with TSG101. The interaction of TSG101 with the M1 protein presumably occurs initially inside the cell. Our present evidence that TSG101 antibody treatment can decrease viral release suggests that these antibodies compete with this interaction. An alternative explanation is that the interaction persists in the presence of antibody treatment and that TSG101 inhibits viral release by altering (eg, via steric hindrance) other interactions or functions of TSG101 during the budding process. Future investigation will be required to distinguish between these possible mechanisms.

Two previous reports had linked ESCRT proteins with influenza infection.[40,41] Specifically, these studies suggested that M1 interacted with another host protein, VPS28, to support influenza propagation. However, technical concerns with some of the assay systems prompted a retraction of these reports. Our results indicate that the interaction of TSG101 with M1 is direct and independent of VPS28. Furthermore, overexpression of the *C*-terminal portion of TSG101 can inhibit influenza virus release (Figure 6).

A TSG101-based antibody therapeutic would be predicted to have broad-spectrum applications. This feature is particularly important for viruses such as influenza, which evolve rapidly by mutation and genetic reassortment.[2,42] If TSG101 is essential for the influenza viral life cycle, then TSG101-based therapies would be expected to be applicable to new or uncommon variants of influenza and antiviral drug-resistant strains. Many human and avian virus strains

have become resistant to conventional influenza antiviral drugs.[43–50] The fact that seemingly unrelated viruses, such as HIV and Ebola viruses, utilize TSG101 in a manner analogous to our present findings with influenza suggests that these viruses have not evolved alternative mechanisms to facilitate viral budding. This raises an intriguing question of whether TSG101-independent viral variants could arise. Unlike conventional antiviral drugs that directly target the pathogen, TSG101-based antivirals should not impart selective pressure on the pathogen, but rather on the host cell, as it is generally understood that the highly plastic nature of viral genome is a primary driver of drug resistance.

Acknowledgments

The authors thank Drs Y Abdul, S Cohen, and H Mao for helpful comments and constructive review. We thank Dr Eric Freed (National Cancer Institute) for the use of TSG-3′ dominant negative mutant. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry. This work is supported by a grant from the United States Army (W81XWH-06-1-0730).

Disclosure

The authors report no conflicts of interest in this work.

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