Human parainfluenza virus type 2 V protein inhibits and antagonizes tetherin

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Tetherin (BST-2/CD317/HM1.24) is an antiviral membrane protein that prevents the release of enveloped viruses from the cell surface. We found that the growth of human parainfluenza virus type 2 (hPIV-2), but not that of V protein-deficient recombinant hPIV-2, was inhibited by tetherin. V protein immunoprecipitates with tetherin, and this interaction requires its C-terminal Trp residues. The glycosyl phosphatidylinositol attachment signal of tetherin, but not its cytoplasmic tail, was necessary for its binding with V. The distribution of the V protein clearly changed when co-expressed with tetherin in plasmid-transfected cells. hPIV-2 infection of HeLa cells reduced cell surface tetherin without affecting total cellular tetherin. This reduction also occurred in HeLa cells constitutively expressing V, whereas mutated V protein did not affect the cell surface tetherin. Our results suggest that hPIV-2 V protein antagonizes tetherin by binding it and reducing its presence at the cell surface.

INTRODUCTION

Human parainfluenza virus type 2 (hPIV-2), a Rubulavirus in the family Paramyxoviridae, is the second cause of severe respiratory tract disease in infants after human respiratory syncytial virus. The hPIV-2 genome encodes seven proteins (NP, V, P, M, F, HN and L proteins) from six genes (Lamb & Parks, 2013). The P/V gene expresses two proteins. V is expressed from the unedited mRNA, whereas P is expressed from an edited mRNA. V and P thus contain a common N-terminal region (Ohgimoto et al., 1990). The unique C-terminal region of V contains seven invariant cysteine residues and three tryptophan residues that are highly conserved in the Paramyxoviridae (Paterson et al., 1995; Lamb & Parks, 2013). Although V is not essential for virus replication, it greatly influences viral growth efficiency (Nishio et al., 2005).

The hPIV-2 V protein interacts with several host cell proteins, such as MDA-5 (Andrejeva et al., 2004; Childs et al., 2007, 2009), LGP2 (Childs et al., 2012), TRAF6 (Kitagawa et al., 2013), STATs (Nishio et al., 2001, 2005; Parisien et al., 2001) and AIP1/Alix (Nishio et al., 2007). TRAF6, STATs and AIP1/Alix are recognized by the C-terminal V-specific region of V (Nishio et al., 2005, 2007; Kitagawa et al., 2013). Whereas the V proteins of avulavirus Newcastle disease virus and rubulaviruses PIV-5 and SV41, and mumps virus target STAT1 for degradation (Didcock et al., 1999; Kubota et al., 2001; Nishio et al., 2001; Andrejeva et al., 2002; Huang et al., 2003), the hPIV-2 V protein degrades STAT2 (Nishio et al., 2001; Parisien et al., 2001). Most host proteins found to bind V are involved in IFN induction and signalling. One of the main functions of V appears to be to promote viral growth by counteracting the host innate immune response.
Fig. 1. Virus growth of hPIV-2 or rPIV-2 carrying V protein mutations in the HeLa cell knocked-down endogenous tetherin. (a) The cell lysates from HeLa cell knocked-down endogenous tetherin (TKD) and control cells (ctrl) were subjected to IB: anti-Bst2, IB: anti-actin, IB: anti-NP, and IB: anti-actin. (b) Tetherin showed a significant decrease in cell lysates from the TKD cells compared to the ctrl cells. (c) p.f.u. ml⁻¹ was measured for each construct, with the TKD showing a significant decrease in viral replication compared to the ctrl. (d) The cell lysates from HeLa cell knocked-down endogenous tetherin (TKD) and control cells (ctrl) were subjected to IB: anti-NP and IB: anti-actin. (e) The amino acid sequences of the V proteins are presented. (f) wt hPIV-2 and rPIV-2 V_C193/197A, rPIV-2 V_C209/211/214A, rPIV-2 V_C218/221A, and rPIV-2 V_W178H/W182E/W192ArPIV-2/P-edit were tested for virus growth in HeLa cells. (g) The ratio of TKD/ctrl is presented for each construct, with the TKD showing a significant decrease in viral replication compared to the ctrl.
immunoblot (IB) analysis using anti-Bst2 polyclonal antibody. Expression of actin in cell lysates was used as a loading control. (b) HeLa-TKD and HeLa-ctrl were subjected to flow cytometry using anti-human CD317 mAb for the detection of cell surface tetherin. HeLa-ctrl and HeLa-TKD are indicated by grey shading and black lines, respectively. Cells not treated with a secondary antibody are indicated by a dashed line. (c) HeLa-TKD or HeLa-ctrl cells were infected with hPIV-2 at an m.o.i. of 1 for 30 h and titres were measured by plaque assay as described in Methods. The p.f.u. values are presented as the mean from five independent experiments. Significance is indicated as $P<0.05$ (*). Error bars indicate s.d. (d) HeLa-TKD and HeLa-ctrl cells were infected with hPIV-2 under the same conditions as in (c). These cell lysates were subjected to IB analysis using anti-NP mAb. Expression of actin in cell lysates was used as a loading control. (e) Amino acid sequences of the V-specific regions of hPIV-2 V protein and its mutants used in this study are shown. Trp and Cys residues are marked with filled circles and squares, respectively. Underlined residues indicate the sites of mutation in each mutant. (f) Cells were infected with wt hPIV-2, rPIV-2/P-edit, rPIV-2/V C193/197A, rPIV-2/V C209/211/214A, rPIV-2/V C218/221A or rPIV-2/V W178H/W182E/W192A at an m.o.i. of 0.01 for 48 h and titres were determined as described in (c). The p.f.u. values are presented as the mean from five independent experiments. Error bars indicate s.d. (g) Results in (f) are represented as ratios of the titres of virus from HeLa-TKD cells to those from HeLa-ctrl cells. The dashed line indicates a TKD/ctrl ratio of 1. Significance is indicated as $P<0.05$ (*). Error bars indicate s.d.

While each of these viruses has its own strategy to counteract tetherin, the strategy most commonly identified is the removal of tetherin from the cell surface, as shown for Vpu of HIV-1 (Neil et al., 2008; Van Damme et al., 2008), Env of HIV-2 (Le Tortorec & Neil, 2009), Nef of SIV (Jia et al., 2009; Zhang et al., 2009; 2011) and K5 of KSHV (Mansouri et al., 2009). Other viral proteins, including GP of Ebola virus (Kaletsy et al., 2009), NS1 and NA of IAV (Mangeat et al., 2012), and glycoproteins (F and HN) of SeV (Bampi et al., 2013) were also identified as tetherin antagonists. However, their mechanism of antagonism is unknown.

In the present study, we analysed the interaction between tetherin and hPIV-2. Our results suggest that the hPIV-2 V protein interferes with the antiviral activity of tetherin by binding it and thereby reducing it at the cell surface.

**RESULTS**

**Knockdown of endogenous tetherin enhances the production of infectious hPIV-2**

HeLa cells express endogenous tetherin, and HeLa-TKD cells, in which the tetherin is knocked down, have decreased total and cell surface expression (Fig. 1a, b). hPIV-2 was inoculated into HeLa-TKD cells and control HeLa cells, HeLa-ctrl, at an m.o.i. of 1. Plaque assays showed that the virus titre from HeLa-TKD was approximately 10-fold higher than that from HeLa-ctrl (Fig. 1c). As the amounts of NP protein expressed in these infected cells were similar (Fig. 1d), tetherin knockdown clearly enhanced production of infectious hPIV-2.

As the hPIV-2 V protein influences viral growth efficiency and tetherin has an antiviral activity, there is likely to be an association between V and tetherin. HeLa-TKD cells were therefore infected with rPIV-2/P-edit, which does not express a V protein, at an m.o.i. of 0.01 for 48 h. While wt hPIV-2 growth was increased more than 10-fold by tetherin knockdown, the growth of rPIV-2/P-edit was not affected by tetherin knockdown (Fig. 1f, g). The C terminus of the V protein-specific region contains functionally important groups of Cys (zinc finger) and Trp residues (Fig. 1e). In order to examine the involvement of these residues, rPIV-2s carrying mutant V proteins (C193/197A, C209/211/214A, C218/221A and W178H/W182E/W192A) were tested (Fig. 1e). Each V-mutated rPIV-2 showed a similar growth pattern regardless of the expression level of tetherin (Fig. 1f, g).

**hPIV-2 V protein binding to tetherin requires its C-terminal Trp-rich motif**

As wt hPIV-2 carrying wt V protein appeared sensitive to tetherin (Fig. 1c, f, g), we examined whether V might directly interact with tetherin by immunoprecipitation (IP). The hPIV-2 V or P genes were transfected into COS cells together with tetherin. Only V co-immunoprecipitated with anti-Bst2 (Fig. 2b, lane 5 of the third panel). This interaction was confirmed using a reverse combination of antibodies (Fig. 2b, lane 5 of the fifth panel). The same results were obtained when using FLAG-tagged tetherin (Fig. 2c, lane 4 of the third panel). Since P and V share the N-terminal 164 aa, the C-terminal V-specific region appears to be necessary for tetherin interaction. To determine the amino acids essential for the interaction, Cys mutants, C193/197A, C209/211/214A and C218/221A, and a Trp mutant, W178H/W182E/W192A, were analysed (Fig. 1e and 2d). IP analysis revealed that replacement of three Trp residues eliminated V binding, while all the Cys mutants continued to bind tetherin (Fig. 2d, lanes 4–8 of the third panel). V binding to tetherin thus requires its C-terminal Trp-rich motif.

**Tetherin C-terminal GPI anchor attachment signal is necessary for its binding to V**

As shown in Fig. 3(a), tetherin has a unique structure consisting of four domains, N-terminal cytoplasmic tail (CT), a transmembrane domain (TM), an extracellular domain (ECD), and a glycosyl phosphatidylinositol (GPI) membrane anchor (Kupzig et al., 2003). We generated four deletion mutants of tetherin with N-terminal FLAG tags

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These mutants consist of residues 22–180 aa (ΔCT-tetherin), 47–180 aa (ΔTM-tetherin), 1–46 aa (ΔECD-tetherin), and 1–161 aa (ΔGPI-tetherin), respectively. V and full-length tetherin or various truncated tetherins (Fig. 3b) were expressed in COS cells and the cell lysates were immunoprecipitated with anti-FLAG. As shown in Fig. 3(c), ΔCT- and ΔTM-tetherin were co-precipitated, while ΔECD- and ΔGPI-tetherin were not. The GPI anchor attachment signal is thus necessary for V binding.

**The hPIV-2 V protein co-localizes with tetherin**

To investigate the subcellular distribution of V and tetherin, immunofluorescent staining assay was carried out. When...
The hPIV-2 V protein decreases HeLa cell surface tetherin

Bampi et al. (2013) reported that SeV, another paramyxovirus, degrades endogenous tetherin in HeLa cells. However, when HeLa cells were infected with hPIV-2 at an m.o.i. of 0.01 or 1, the amount of tetherin at 24 h post-infection (p.i.) measured by immunoblot (IB) analysis was similar to that of non-infected cells (Fig. 5a), indicating that hPIV-2 infection does not affect the total amount of cellular tetherin. Since tetherin inhibits virus release from the cell surface, we next measured the expression of cell surface tetherin using flow cytometry. At 24 h p.i. with hPIV-2 at m.o.i. of 0.01 and 1, non-permeabilized HeLa cells were subjected to FACs (fluorescence-activated cell sorting) analysis. The main peak of the histogram of cells infected with wt hPIV-2 showed a significant leftwards shift (less cell-surface expression) relative to that of control cells (Fig. 5b, c). Even cells infected with hPIV-2 at a low m.o.i. showed a reduction of cell surface tetherin. A minor peak in the histogram of cells infected at a low m.o.i. is probably due to uninfected cells (Fig. 5b). To examine the involvement of V protein, the amounts of total tetherin and surface tetherin of rPIV-2/P-edit-infected cells were compared with those of hPIV-2 infected cells (Fig. 5a–c). Unlike wt hPIV-2, rPIV-2/P-edit infection did not affect either total or surface tetherin. These results suggest that the hPIV-2 V protein negatively regulates the cell surface tetherin.

To confirm tetherin cell surface downregulation by the hPIV-2 V protein, we compared HeLa cells constitutively expressing V, V_{W178H/W182E/W192A} or P with normal HeLa cells. The total amount of tetherin in all these cells was similar to that in normal HeLa cells (Fig. 5d). The cell surface expression of tetherin in these cells was also measured using FACs analysis. As shown in Fig. 5(e, f), there was a significant leftward shift in the histogram of HeLa/V cells, but not of HeLa/V_{W178H/W182E/W192A} and HeLa/P cells. The total amount and surface expression of tetherin in other HeLa/V Cys-mutants were also similar to those in normal HeLa cells (data not shown). These data collectively suggest that the C-terminal region of hPIV-2 V protein is required for the reduction of the cell surface tetherin, without its degradation.

**DISCUSSION**

Since each enveloped virus has a unique way of interacting with tetherin, understanding the virus–tetherin interaction requires individual investigation with each virus. As shown in Fig. 1(c, d), tetherin negatively regulated the growth of hPIV-2 without affecting its protein synthesis. For hPIV-2, the V protein appears to be the tetherin antagonist, as might be expected (Nishio et al., 2001, 2005, 2007; Kitagawa et al., 2013). V can bind to tetherin (Fig. 2b, c), and the tryptophan-rich motif in the C-terminal V-specific region, but not the Cys residues of the zinc finger, is
important for this direct interaction (Fig. 2d). However, all these residues are required for V to act as a tetherin antagonist, which mirrors the situation with STAT degradation (Nishio et al., 2005). rPIV-2s with Cys- or Trp-mutated V proteins could not reduce the cell surface expression of tetherin (Fig. 5e and data not shown); i.e. simply binding the host protein is necessary but not sufficient to inhibit its activity. No influence of tetherin knockdown on the growth of V-deficient and -mutated rPIV-2s (Fig. 1f, g) is apparently inconsistent with our claim that V antagonizes tetherin. Drastic inhibition of their growth by the failure to counteract the host immune system might mask the effect of tetherin on their growth. V proteins of other rubulaviruses, such as PIV-5, mumps virus, SV41 and hPIV-4A, contain three Trp residues (Trp-Trp-Trp or Tyr-Trp-Trp) at their C-termini, which were previously reported to be important in binding STATs, and inducing their degradation (the hPIV-4A V protein is an exception). Thus, it is likely that these V proteins also bind to tetherin and antagonize it.

Viral tetherin antagonists are transmembrane proteins (Vpu of HIV-1, Env of HIV-2, GP of Ebola virus, K5 of KSHV) or a membrane-associated protein (Nef of SIV). Two IAV proteins, NS1 and NA, have the potential to counteract tetherin (Mangeat et al., 2012). NS1 is the only non-membrane protein reported to be a tetherin antagonist, but IAV tetherin antagonism is controversial (Watanabe et al., 2011; Mangeat et al., 2012; Winkler et al., 2012). We at first expected that tetherin would bind V via its CT domain, since the V protein is present in the cytoplasm and/or nucleus. Surprisingly, deletion of the GPI attachment signal eliminated binding to V, and mutants lacking CT did not affect this interaction (Fig. 3c). This suggests that the hPIV-2 V antagonizes tetherin differently from the other viral antagonists. If the V protein directly binds to the GPI attachment signal of tetherin, their interaction would need to occur during the short period before the GPI attachment signal is removed. Furthermore, the GPI attachment signal is present in the lumen of the endoplasmic reticulum (Kinoshita et al., 1997), to which V does not have access. Therefore, this interaction may be indirect, mediated through a cellular protein, particularly a transmembrane protein. Andrew et al. (2011) reported that tetherin contains a second TM region instead of a GPI anchor. If so, V protein would directly bind to a short cytoplasmic region at the tetherin C terminus.

Tetherin inhibits virus release from the plasma membrane, and viruses must counteract cell surface tetherin for effective growth. We have found that the hPIV-2 V protein reduced cell surface tetherin without affecting total cellular tetherin, in both infected and V-expressing HeLa cells (Fig. 5). Reduction of cell surface tetherin is also caused...
Fig. 5. Effects of hPIV-2 infection and the V protein on the level of tetherin in HeLa cells. (a) HeLa cells were infected with wt hPIV-2 or rPIV-2/P-edit at the indicated m.o.i. for 24 h and subjected to IB analysis using the indicated antibodies. Actin was measured as a loading control. The histogram shows quantitative densitometry of tetherin analysed using ImageJ software (http://rsb.info.nih.gov/ij). The relative value for the mock infection equals 1 (dashed line). Error bars indicate sd. (b) HeLa cells were infected with wt hPIV-2 or rPIV-2/P-edit under the same conditions as in (a), and subjected to flow cytometry using anti-human CD317 mAb for the detection of cell surface tetherin. Cells not infected with hPIV-2 and hPIV-2-infected cells are indicated by grey shading and black lines, respectively. (c) The geometric mean fluorescence intensity (MFI) of tetherin is shown. Cells not treated with a secondary antibody were used as a negative control. Significance is indicated as P<0.05 (*). Error bars indicate sd. (d) Normal HeLa and V-, mutated V-, or P-expressing HeLa cells were lysed and subjected to IB analysis with the indicated antibodies. The histogram shows quantitative densitometry of tetherin analysed as described in (a). (e) Cells as described in (d) were subjected to FACS analysis using anti-CD317 mAb. Normal HeLa cells and V-, mutated V-, or P-expressing HeLa cells are indicated by grey shading and black lines, respectively. (f) The MFI of tetherin is shown as described in (c). All experiments were performed at least three times independently.

by other tetherin antagonists, namely HIV-1 Vpu (Neil et al., 2008; Van Damme et al., 2008), KSHV K5 (Mansouri et al., 2009), HIV-2 Env (Le Tortorec & Neil, 2009) and SIV Nef (Jia et al., 2009; Zhang et al., 2009, 2011). These antagonists are mainly divided into two groups by their detailed mechanisms; the first two degrade tetherin via the endosome/lysosome pathway, and the latter two internalize it through clathrin-dependent endocytosis. Our results indicated that hPIV-2 V protein removed tetherin from the cell surface and/or inhibited its transport to the cell surface via a tetherin degradation-independent pathway. As shown in Fig. 4, the expression of hPIV-2 V protein did not significantly induce perinuclear accumulation of tetherin like HIV-2 Env (Le Tortorec & Neil, 2009). However, the distribution of hPIV-2 V protein was affected by the tetherin expression. Since hPIV-2 V protein co-localized with tetherin in the cytoplasm, hPIV-2 V protein might interact with internalized or newly synthesized tetherin and thereby prevent its transport to the cell surface.

Recently, several groups have reported that tetherin also acts as a viral sensor and an effector that regulates the innate immune response, as well as restricting virus budding (Galão et al., 2012; Tokarev et al., 2013). They reported that tetherin induces NF-κB together with other molecules such as TAK1, TABs and TRAFs. TRAF6 particularly seems to be essential for efficient signalling leading to NF-κB activation and proinflammatory gene expression. Tetherin reportedly interacts with TRAF6, although whether the interaction is direct is unknown (Galão et al., 2012). Since we previously reported that hPIV-2 V protein also co-immunoprecipitates with TRAF6 (Kitagawa et al., 2013), V protein might also inhibit the role of tetherin as a virus sensor.

In conclusion, we have provided evidence that the hPIV-2 V protein binds to tetherin and reduces its amount at the cell surface. The poor growth of V-deficient rPIV-2 in several cell lines might be due not only to the failure to inhibit IFN signalling, but also to the loss of direct tetherin antagonism. Further studies are needed to uncover the detailed mechanism(s) of how V inhibits tetherin function.

METHODS

Cells and viruses. Vero cells were grown in Eagle’s minimal essential medium (MEM) supplemented with 10 % FCS. COS cells, HeLa cells, and their derivatives were grown in Dulbecco’s modified Eagle’s MEM (DMEM) containing 10 % FCS. A tetherin knockdown HeLa cell line (HeLa-TKD) using a tetherin shRNA-containing plKO and its control cell line (HeLa-ctrl) transduced with an empty vector were kind gifts from Dr J. Yasuda (Nagasaki University, Japan). HeLa/V, HeLa/ V_W178H/W182E/W192A, HeLa/V_C193/197A and HeLa/P cells, which stably express hPIV-2 V, V mutants or P protein were previously described (Nishio et al., 2001, 2005). All cells were maintained in a humidified incubator at 37 °C with 5 % CO₂. hPIV-2 (Toshiba strain), V-deficient recombinant hPIV-2 (rPIV-2/P-edit), and V-mutated recombinant hPIV-2s were used in this study (Nishio et al., 2005).

Antibodies. mAbs against hPIV-2 V/P protein (315-1) and NP protein (159-1) were described previously (Nishio et al., 1997, 1999). mAb and polyclonal antibody to FLAG were obtained from Sigma. mAb to actin (sc-8432) was purchased from Santa Cruz Biotechnology. The anti-tetherin mAb used in this study was anti-human CD317 (BioLegend). The polyclonal anti-tetherin antibody was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: anti-Bst2 from Drs Klaus Strebel and Amy Andrew (https://www.aidsreagent.org/index.cfm) (Miyaig et al., 2009).

Plasmids. hPIV-2 genes of V, P, NP and V mutants were cloned into the mammalian expression vector pcDNA3.1(−), as described previously (Nishio et al., 1996, 1997, 2005). An expression vector, pEF-BOS-EX, harbouring tetherin cDNA was kindly provided by Dr J. Yasuda. cDNA of tetherin and its deletion mutants were fused to FLAG tag at the N terminus and ligated into another expression vector, pcDNA3.1(−) (Invitrogen). All of these constructs were confirmed by DNA sequencing.

Virus titration. Monolayers of HeLa-TKD or HeLa-ctrl cells were infected with hPIV-2, rPIV-2/P-edit or V-mutated rPIV-2s at an m.o.i. of about 1 or 0.01 and incubated in MEM without FCS. At 30 or 48 h p.i., virus titres were measured by plaque assay using Vero cells as described previously, with a little modification (Nishio et al., 2005). Briefly, 300 μl of each serial 10-fold dilution of the virus solutions in MEM without FCS was added to each well of a 12-well plate with Vero cells. After 1 h of adsorption, virus solutions were removed and MEM containing 2 % FCS and 1 % SeaKem ME agarose (FMC BioProducts) was added. At 5 days p.i., the cells were stained with 0.1 % neutral red and the number of plaques (p.f.u.) was counted.

IB and IP analysis. COS cells in six-well plates were transfected with plasmids encoding tetherin or its mutants, and various viral proteins...
using XtremeGENE 9 (Roche), according to the manufacturer’s procedures. At 48 h post-transfection (p.t.), cells were harvested, sonicated for 30 s three times in ice-cold lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 0.6 % NP-40, and centrifuged. IB was performed as described previously (Nishio et al., 2002). For IP, cell lysates were incubated with appropriate antibodies overnight at 4 °C. They were reacted with nProtein A Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences) for 3 h at 4 °C and washed three times with PBS. Then, proteins were separated from Sepharose beads with sample buffer for SDS-PAGE, followed by SDS-PAGE and IB analysis.

**Immunofluorescence assay.** HeLa cells grown to 50 % confluence in 12-well plates were transfected with indicated plasmids using XtremeGENE HP (Roche) according to the manufacturer’s procedures. At 48 h p.t., the cells were fixed with 4 % paraformaldehyde for 15 min at room temperature and rinsed three times with PBS. The cells were permeabilized with PBS containing 0.3 % (w/v) Triton X-100 for 5 min and washed three times with PBS. The cells were incubated for 60 min with anti-FLAG and mAb against V/P protein and washed three times with PBS. The secondary antibody reaction was performed for 60 min with AlexaFluor 488 goat anti-mouse IgG and AlexaFluor 594 goat anti-rabbit IgG (Invitrogen). After washing the cells with PBS, they were analysed with a fluorescence microscope.

**Flow cytometry.** Cells grown in 12-well or 24-well plates were harvested with MEM containing 0.53 mM EDTA and 0.05 % trypsin. The following procedures for immunostaining were performed on ice. After washing three times with PBS containing 2 % FCS and 0.1 % sodium azide, cells were reacted with anti-CD317 mAb for 30 min on ice. Cells were incubated with AlexaFluor 488 goat anti-mouse IgG (Invitrogen) for 30 min. Infected cells were fixed with 1 % paraformaldehyde. Cells were then analysed with a FACScalibur cytometer (BD Biosciences).

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