Membrane-anchored human Rab GTPases directly mediate membrane tethering in vitro

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ABSTRACT
Rab GTPases are master regulators of eukaryotic endomembrane systems, particularly functioning in membrane tethering to confer the directionality of intracellular membrane trafficking. However, how exactly Rab GTPases themselves act upon membrane tethering processes has remained enigmatic. Here, we thoroughly tested seven purified Rab GTPases in human, which localize at the various representative organelles, for their capacity to support membrane tethering in vitro. Strikingly, we found that three specific human Rabs (endoplasmic reticulum/Golgi Rab2a, early endosomal Rab5a, and late endosomal/lysosomal Rab7a) strongly accelerated membrane aggregation of synthetic liposomes even in the absence of any additional components, such as classical tethers, tethering factors, and Rab effectors. This Rab-induced membrane aggregation was a reversible membrane tethering reaction that can be strictly controlled by the membrane recruitment of Rab proteins on both apposing membranes. Thus, our current reconstitution studies establish that membrane-anchored human Rab GTPases are an essential tethering factor to directly mediate membrane tethering events.

KEY WORDS: Rab GTPase, Liposome, Membrane tethering, Membrane traffic, Reconstitution

INTRODUCTION
Eukaryotic cells organize and maintain the complex but highly specific secretory and endocytic trafficking pathways to deliver correct sets of cargo molecules towards their various subcellular organelles and plasma membranes (Bonifacino and Glick, 2004). These membrane trafficking events are temporally and spatially regulated by a variety of key protein components, including SNARE proteins (Jahn and Scheller, 2006), SNARE-binding cofactors such as Sec1/Munc18 proteins (Rizo and Südhof, 2012), Rab GTPases (Stenmark, 2009), and Rab-interacting effector proteins (Grosshans et al., 2006). Membrane tethering, the first contact of organelles and transport vesicles before membrane docking and fusion, is a critical step to control the directionality of membrane traffic and has been proposed to be mediated by docking and fusion, is a critical step to control the directionality of intracellular membrane trafficking. However, as Rab2a-His12 showed significant differences from the other Rab-His12 proteins in the CD spectra and predicted secondary structure contents, it should be noted that our preparation of Rab2a-His12 likely contained some denatured or partially-denatured proteins.

RESULTS AND DISCUSSION
Rab GTPases are typically post-translationally modified by an isoprenyl lipid group at their C-terminal cysteine residues, which is required for membrane association of Rabs (Hutagalung and Novick, 2011). To mimic the membrane-bound state of native Rabs bearing the lipid anchor, the seven selected human Rabs were purified as the C-terminal polyhistidine-tagged forms (Rab-His12 proteins) that can be attached to liposome membranes bearing a DOGS-NTA lipid (1,2-dioleoyl-sn-glycero-3-\([-\{N-(5-amino-1-carboxypentyl)iminodiacetic acid\}-sucryl\] (Fig. 1A, lanes 1–7). For a negative control, we also purified the His12-tagged form of human HRas, which is a similar Ras-family GTPase with a C-terminal lipid anchor but not functionally related to membrane tethering events (Fig. 1A, lane 8). All the purified Rab-His12 and HRA-His12 proteins retained their intrinsic GTP-hydrolysis activities, specifically converting GTP to GDP and a free phosphate group (Fig. 1B,C). In addition, we further characterized the purified Rab proteins by circular dichroism (CD) spectroscopy (Fig. 2). The six Rab-His12 proteins tested, except Rab2a-His12, had similar far-UV CD spectra (Fig. 2) and comparable secondary structure contents which were estimated from the CD spectra using a K2D3 program (Table 1) (Louis-Jeune et al., 2012). These biochemical properties support that those six Rab-His12 proteins are a well-folded protein that indeed has the capacity to bind and hydrolyze a guanine nucleotide. However, as Rab2a-His12 showed significant differences from the other Rab-His12 proteins in the CD spectra and predicted secondary structure contents, it should be noted that our preparation of Rab2a-His12 likely contained some denatured or partially-denatured proteins.

Three selective Rab GTPases specifically promote robust liposome aggregation
Using purified Rab-His12 proteins, two types of liposomes that bore DOGS-NTA and either biotin-labeled phosphatidylethanolamine (biotin-PE) or rhodamine-labeled PE (Rh-PE), and streptavidin-coated beads, we developed an in vitro assay to test whether membrane-bound Rabs promote liposome aggregation (Fig. 3A). Reaction mixtures containing those two distinct liposomes decorated with Rab-His12 proteins were incubated with streptavidin beads to isolate the biotin-PE liposomes, followed by
measuring Rh fluorescence for quantifying the amounts of the Rh-PE liposomes co-isolated with the biotin-PE liposomes (Fig. 3A). Strikingly, three specific Rabs (Rab2a at endoplasmic reticulum (ER)/Golgi, Rab5a at early endosomes, and Rab7a at late endosomes or lysosomes) supported stable association of the Rh-PE liposomes with the biotin-PE liposomes, whereas the other four Rabs (Rab1a, Rab3a, Rab4a, and Rab6a) and HRas had little effect on assemblies of these liposomes (Fig. 3B). However, even those three active Rabs (Rab2a, Rab5a, and Rab7a) were not able to initiate efficient assemblies of highly curved, small liposomes prepared by extrusion through a 100-nm pore filter (Fig. 3C), in contrast to relatively large-size liposomes extruded through a 400-nm or 1000-nm filter (Fig. 3B,D). This reflects that the robust activities for the three Rabs to promote liposome assemblies are dependent on the size of liposomes used. Membrane tethering of small highly-curved vesicles may require the other additional factors that sense membrane curvature, as previously reported for human golgin GMAP-210, the Golgi-associated coiled-coil protein which contains an ALPS (amphipathic lipid-packing sensor) motif (Drin et al., 2007; Drin et al., 2008).

To further characterize the Rab-induced liposome assemblies, we employed turbidity assays of liposome suspensions in the presence of Rab proteins (Fig. 3E,F). In accord with the results in streptavidin-bead assays (Fig. 3B), the same three specific Rabs (Rab2a, Rab5a, and Rab7a) caused robust increases in the turbidity of liposome suspensions (Fig. 3E). In particular, Rab5a and Rab7a strongly accelerated the initial rates of the turbidity increases as in panel B, but in the presence of GTP (1 mM), GDP (1 mM), or GTP:γS (1 mM), where indicated.

Table 1. Predicted secondary structure contents of purified Rab-His12 proteins

| Rab proteins | α-helix (%) | β-strand (%) |
|--------------|-------------|--------------|
| Rab1a-His12  | 24.0        | 24.6         |
| Rab2a-His12  | 14.6        | 34.2         |
| Rab3a-His12  | 29.7        | 19.9         |
| Rab4a-His12  | 23.8        | 27.8         |
| Rab5a-His12  | 25.1        | 23.1         |
| Rab6a-His12  | 22.0        | 24.0         |
| Rab7a-His12  | 28.6        | 22.2         |

1Secondary structure contents were estimated from far-UV CD spectra, using a K2D3 program (Louis-Jeune et al., 2012).
Fig. 3. Three human Rab GTPases are specific proteins to drive liposome aggregation. (A) Schematic representation of the liposome aggregation assay using streptavidin-coated beads, two types of liposomes bearing either biotin-PE/DOGS-NTA/FL-PE or Rh-PE/DOGS-NTA, and purified Rab-His12 proteins. (B–D) Rab2a, Rab5a, and Rab7a promote robust liposome aggregation. The Rh-labeled liposomes (1.5 mM lipids) were mixed with the biotin-labeled liposomes (1.8 mM lipids), Rab-His12 proteins (4 μM), and streptavidin beads, and incubated (30˚C, 2 hours). The Rh-labeled liposomes co-isolated with streptavidin beads were analyzed by measuring the Rh fluorescence. Liposomes were prepared by extrusion through 400 nm (B), 100 nm (C), or 1000 nm (D) filters. AU, arbitrary units. (E,F) Kinetics of Rab-induced liposome aggregation. To monitor turbidity changes of liposome suspensions with Rabs, liposomes (1.3 mM lipids) were mixed with Rab-His12 proteins [2 μM (E), 0.5–2 μM (F)], followed by measuring the absorbance at 400 nm. (G–L) Rab2a, Rab5a, and Rab7a induce the formation of massive liposome clusters. As represented in panel G, the FL-PE liposomes (1.8 mM lipids) and Rh-PE liposomes (1.5 mM lipids) were mixed without Rabs (H) or with Rab1a-His12 (I), Rab2a-His12 (J), Rab5a-His12 (K), and Rab7a-His12 (L) (4 μM each). After incubation (30˚C, 2 hours), fluorescence images of the liposome suspensions were obtained. Scale bars: 5 μm.
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nucleotide-preloaded Rabs, the Rab-specific guanine nucleotide

Rab7a (Fig. 4A,B). Further reconstitution studies with guanine

reactions by the three active Rab GTPases, Rab2a, Rab5a, and

GTPases to promote membrane aggregation, and also that GDP

restore or further stimulate the capacity of the human Rab

experimental conditions, we observed that added GTP did not

aggregation without adding guanine nucleotides (Fig. 3).

Notably, when GTP and GDP were exogenously added in the

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bound states and thereby interact with their specific Rab effectors

for the downstream functions, including membrane tethering and
docking (Grosshans et al., 2006; Stenmark, 2009; Hutagalung and

Novick, 2011). Moreover, the prior in vitro analyses of yeast

Rabs indicated that the tethering activity of the yeast Rab5/

Vps21p relied on its GTP-loaded form (Lo et al., 2012). In this

context, we asked whether guanine nucleotides are an essential

component in in vitro membrane aggregation mediated by human

Rabs (Fig. 4), even though we had observed that at least three

Rabs (Rab2a, Rab5a, and Rab7a) initiated robust lipidosome

aggregation without adding guanine nucleotides (Fig. 3). Notably,

when GTP and GDP were exogenously added in the streptavidin bead assays and turbidity assays, these nucleotides

had no significant effect on the Rab-dependent lipidosome

aggregation reactions (Fig 4A,B). Under these current

experimental conditions, we observed that added GTP did not

restore or further stimulate the capacity of the human Rab

GTPases to promote membrane aggregation, and also that GDP

addition had no inhibitory effect on lipidosome aggregation reactions by the three active Rab GTPases, Rab2a, Rab5a, and

Rab7a (Fig. 4A,B). Further reconstitution studies with guanine

nucleotide-preloaded Rabs, the Rab-specific guanine nucleotide

exchange factors, and the Rab GTPase-activating proteins will be

required to more thoroughly assess the GTP/GDP-dependence of

Rab-mediated membrane aggregation.

Membrane-anchored Rab proteins specifically mediate

reversible membrane tethering reactions

We next tested whether membrane attachment of Rab proteins is

indeed indispensable for their specific function to cause membrane

aggregation (Fig. 5). Liposome co-sedimentation assays confirmed

that Rab-His12 proteins were stably bound to the DOGS-NTA-

containing liposomes (Fig. 5A, lanes 1 and 4) and that the

membrane attachment of Rabs was fully abolished when used the

liposomes lacking DOGS-NTA or the untagged Rabs without a C-
terminus His12 tag instead (Fig. 5A, lanes 2, 3, 5, and 6).

Strikingly, Rab5a and Rab7a completely lost their potency to

initiate lipidosome aggregation under those conditions where Rabs

no longer stably associated with lipidosomal membranes (Fig. 5B–

D). Moreover, we found that Rab5a and Rab7a had to be anchored

on both, not either one, of two opposing lipidosomal membranes

for driving membrane aggregation (Fig. 5B, lanes 3 and 7; Fig. 5C),

suggesting that Rab-induced membrane aggregation is promoted

by trans-Rab protein assemblies on apposed membranes. Next,

to test whether Rab-mediated lipidosome aggregation can be

competitively blocked by addition of untagged Rab proteins,

which have no C-terminal His12 tag for membrane attachment but

may be able to associate with membrane-anchored Rab-His12

proteins, we employed the turbidity assays in the presence of both

Rab5a-His12 and untagged Rab5a (Fig. 5E). However, even at 8-

fold molar excess of untagged Rab5a over Rab5a-His12, the

soluble untagged Rab5a protein had little inhibitory effect on

Rab5a-mediated lipidosome aggregation (Fig. 5E). This may reflect

that membrane-anchored Rab5a exclusively recognize and

assemble in trans with Rab5a on opposing membranes destined
to tether, not membrane-detached soluble Rab5a, thereby

conferring specific membrane tethering events.

We then asked whether the lipidosome aggregation reactions

mediated by membrane-anchored Rab proteins are indeed a

reversible reaction, like physiological membrane tethering events

(Ungermaier et al., 1998). To address this, the pre-formed Rab-

mediated lipidosome aggregates were further incubated with

imidazole and EDTA, which lead to dissociation of Rab5a-His12

from DOGS-NTA-bearing liposomes, and then tested by the

streptavidin-bead assay (Fig. 6A,B) and fluorescence microscopy

(Fig. 6C–E). In these analyses, we observed that the imidazole

and EDTA treatments completely or thoroughly disassembled
the lipidosome aggregates which had been induced by membrane-bound

Rab5a-His12 proteins (Fig. 6B–E). This indicates that the

fig. 4. Rab-induced liposome aggregation in the presence of exogenous guanine nucleotides.

(A) Addition of exogenous guanine nucleotides has no effect on Rab-induced

liposome aggregation.

Liposome aggregation assays were employed as in Fig. 3B,

but in the presence of 1 mM GTP or GDP. (B) Turbidity

changes of liposome suspensions were assayed for

Rab5a-His12 (0.5 μM) as in

Fig. 3E,F, but in the presence of

GTP/GDP.
Fig. 5. Rab-mediated liposome aggregation requires membrane recruitment of Rabs on both apposing membranes. (A) Liposome co-sedimentation assays. The Rh-PE liposomes (1.5 mM lipids) were mixed with Rab proteins (4 μM), incubated (30°C, 2 hours), centrifuged, and analyzed by SDS-PAGE and Coomassie Blue staining. The Rh-PE liposomes lacking DOGS-NTA were used instead where indicated (no DOGS-NTA, lanes 2, 5, 8, and 11). (B) Liposome aggregation was assayed as in Fig. 3B, with Rab5a-His12, Rab7a-His12, heat-treated Rab-His12 proteins (lanes 2 and 6), untagged Rabs (lanes 4 and 8), and a His12 peptide (lane 9). The Rh-PE liposomes lacking DOGS-NTA was used instead where indicated (no DOGS-NTA in Rh-PE liposomes, lanes 3 and 7). (C, D) Fluorescence microscopy was performed as in Fig. 3H–L, with Rab5a-His12, the Rh-PE liposomes lacking DOGS-NTA, and the FL-PE liposomes that bear DOGS-NTA (C) or not (D). (E) Addition of untagged Rab5a does not competitively block Rab5a-induced liposome aggregation. Turbidity changes of liposome suspensions (1.0 mM lipids) were assayed for Rab5a-His12 (1.0 μM) as in Fig. 3E,F; but in the presence of untagged Rab5a (1–8 μM). Scale bars: 5 μm.
Rab-mediated membrane aggregation found here is a reversible process of membrane tethering and can be reversibly regulated by membrane attachment and detachment cycles of Rab proteins. Since several prior studies have demonstrated that the stable membrane attachment of Rab proteins is accompanied by GDP/GTP exchange and facilitated by specific Rab guanine nucleotide exchange factors (Ullrich et al., 1994; Soldati et al., 1994; Gerondopoulos et al., 2012; Blu¨mer et al., 2013), the current results lead us to postulate that the GTP requirement for Rab-mediated tethering is directly linked to membrane recruitment of Rab proteins and thereby can be bypassed by artificially membrane-anchored Rab-His12 proteins on DOGS-NTA-bearing membranes in the present chemically-defined system.

Taken together, the current biochemical analyses using purified human Rab proteins and synthetic liposomes have established that membrane-anchored Rab GTPases have the inherent potency to directly mediate reversible membrane tethering events (Figs 3–6). This conclusion is, however, apparently not compatible with the classical membrane tethering model, in which Rab-interacting coiled-coil tethering factors and/or multisubunit tethering complexes, but not Rab GTPases themselves, function as a key component to directly drive membrane tethering (Pfeffer, 1999; Grosshans et al., 2006; Cai et al., 2007; Yu and Hughson, 2010). This study is also not fully consistent with the recent pioneering work by Merz and colleagues, which reported that only yeast endosomal Rabs such as Vps21p, but not the lysosomal/vacuolar Rab GTPase Ypt7p, can support efficient tethering of liposomes (Lo et al., 2012). Our current findings, therefore, reopen the debate about how Rab GTPases, Rab effectors, and tethering factors work together to mediate specific membrane tethering processes in secretory and endocytic membrane trafficking pathways.

MATERIALS AND METHODS

Protein purification

The coding sequences of full-length human Rabs (Rab1a, Rab2a, Rab3a, Rab4a, Rab5a, Rab6a, and Rab7a) and HRas proteins were amplified by PCR using the Human Universal QUICK-Clone cDNA II (Clontech) as a template cDNA and cloned into a pET-41 Ek/LIC vector (Novagen) expressing a GST-His6-tagged protein. These PCR fragments contained the sequence encoding the protease cleavage site (Leu–Glu–Val–Leu–Phe–Gln–Gly–Pro) for human rhinovirus 3C protease (Novagen) upstream of the initial ATG codons and the sequence encoding the polyhistidine residues (His12) downstream of the codons for a C-terminal residue, to obtain full-length Rab and HRas proteins with only three extra N-terminal residues (Gly–Pro–Gly) and a C-terminal His12-tag after 3C protease cleavage. To prepare the Rab5a and Rab7a proteins lacking a His12-tag (untagged Rab5a and untagged Rab7a; Fig. 1A, lanes 9 and 10, respectively), the PCR fragments without the His12-coding sequence for these Rab proteins were also amplified and cloned into a pET-41 Ek/LIC vector. Recombinant Rab and HRas proteins were produced in the Escherichia coli Rosetta 2(DE3) cells (Novagen) in Terrific Broth medium (1 liter each) with kanamycin (50 µg/ml) and chloramphenicol.
(50 μg/ml) by induction with 0.5 mM IPTG (34°C, 3 hours). E. coli cells were harvested and resuspended in 40 ml each of HN150 (20 mM Hepes-NaOH, pH 7.4, 150 mM NaCl) containing 10% glycerol, 1 mM DTT, 1 mM PMSE, 2.0 μg/ml pepstatin A, and 2 mM EDTA. Cell suspensions were freeze-thawed in a liquid nitrogen bath and a water bath at 25°C, lysed by sonication (UD-201 ultrasonic disruptor; Tomy Seiko, Tokyo, Japan), and centrifuged [50,000 rpm, 75 min, 4°C, 70 Ti rotor (Beckman Coulter)]. GST-His6-3C-tagged Rab and HRas proteins in the supernatants were isolated by mixing with glutathione-Sepharose 4B beads (50% slurry, 2 ml for each; GE Healthcare) and incubating at 4°C for 2 hours with gentle agitation. After washing the protein-bound glutathione-Sepharose 4B beads by HN150 containing 5 mM MgCl2 and 1 mM DTT, purified Rab and HRas proteins were cleaved off and eluted by incubating the beads with human rhinovirus 3C protease (12 units/ml final) in the same buffer (2 ml for each protein) at 4°C.

GTPase activity assay
GTP-hydrolysis activities of recombinant Rab and HRas proteins were assayed by quantitating released free phosphate molecules, using the Malachite Green-based reagent Biomol Green (Enzo Life Sciences). Purified Rab and HRas proteins (4 μM or 6 μM final for each) were incubated at 30°C for 2 hours in HN150 containing MgCl2 (6 mM), DTT (1 mM), and GTP (1 mM), GDP (1 mM), or GTPγS (1 mM) where indicated. The reaction mixtures (100 μl each) were then supplemented with 900 μl of the Biomol Green reagent for each, incubated at 30°C for 20 min or 30 min, and analyzed by measuring the absorbance at 650 nm with a DU720 spectrophotometer (Beckman Coulter). The heat-treated Rab and HRas GTPases that had been denatured by treatment at 100°C for 15 min were also tested with the same protocol. Data obtained in this assay were corrected by subtracting the absorbance value of the control reaction assayed in the absence of Rab and HRas proteins. Means and standard deviations of the corrected values (AA650) were determined from three independent experiments.

CD spectroscopy
Far-UV CD spectra of purified recombinant Rab and HRas proteins were measured with a J-820 spectropolarimeter (Jasco) using a cell with a light path of 0.1 mm. Rab1a-His12 (14 μM), Rab2a-His12 (33 μM), Rab3a-His12 (15 μM), Rab4a-His12 (30 μM), Rab5a-His12 (47 μM), Rab6a-His12 (55 μM), Rab7a-His12 (14 μM), HRas-His12 (16 μM), untagged Rab5a (20 μM), and untagged Rab7a (9.3 μM) were analyzed at 4°C in HN150 containing 10% glycerol, 5 mM MgCl2, and 1 mM DTT. CD signals obtained at 195–250 nm were expressed as the mean residue ellipticity [θ]. Protein secondary structure contents were estimated from CD spectra, using a K2D3 program (Louis-Jeune et al., 2012).

Liposome preparation
Non-fluorescent lipids were from Avanti Polar Lipids. Fluorescent Rh-PE and fluorescein-PE (FL-PE) were from Molecular Probes. Lipid mixtures for the biotin/FL-labeled or Rh-labeled liposomes contained 1-palmitoyl-2-oleoyl phosphatidylcholine (41% mol/mol), POPE (14.5% or 16.5% for the biotin/FL-labeled or Rh-labeled liposomes), soy phosphatidylinositol (10%), PO-phosphatidylserine (5%), cholesterol (20%), DOGS-NTA (6.0%), biotin-PE (2.0% for the biotin/FL-labeled liposomes), and fluorescent lipids (1.5% of FL-PE or Rh-PE for the biotin/FL-labeled or Rh-labeled liposomes). Dried lipid films (8 mM lipids) were hydrated in HN150, incubated (37°C, 30 min), freeze-thawed, and extruded 21 times through polycarbonate filters in a mini-extruder (Avanti Polar Lipids) at 40°C. Lipid concentrations were determined from the fluorescence of FL-PE (λex=495 nm, λem=520 nm) and Rh-PE (λex=560 nm, λem=580 nm).

Liposome aggregation assay using streptavidin-coated beads
Rab-His12 proteins (4 μM) were mixed with the biotin/FL-labeled (1.8 mM lipids) and Rh-labeled (1.5 mM lipids) liposomes in HN150 containing 6 mM MgCl2, 1 mM DTT, and 0.1 mg/ml BSA and incubated with streptavidin-coated beads (Dynabeads M-280 Streptavidin; Invitrogen) (30°C, 2 hours). GTP, GDP, imidazole, EDTA, and a His12 peptide were supplemented where indicated. The streptavidin beads were then washed by HN150 containing 6 mM MgCl2 and 1 mM DTT, resuspended in 100 mM β-OG, and centrifuged. To quantify the co-isolated Rh-labeled liposomes, Rh fluorescence of the supernatants was measured by a SpectraMAX Gemini XPS plate reader (Molecular Devices). Means and standard deviations of the Rh fluorescence signals were obtained from three independent experiments.

Turbidity assay
Turbidity of liposome suspensions was analyzed as described (Ohki et al., 1982). Liposomes (Rh-labeled liposomes, 1.3 mM or 1.0 mM lipids) were mixed with Rabs (0.5–2 μM) in HN150 containing 5 mM MgCl2, 1 mM DTT, 0.1 mg/ml BSA, and 2.5% glycerol, followed by measuring the absorbance at 400 nm at room temperature in a DU720 spectrophotometer (Beckman Coulter).

Fluorescence microscopy
Fluorescence microscopy of liposome suspensions (in HN150 containing 6 mM MgCl2, 1 mM DTT, and 0.1 mg/ml BSA) was performed with a BZ-9000 fluorescence microscope (Keyence) equipped with a Plan Apo VC100×1.4 NA oil irris objective lens (Nikon), using TRITC and GFP-BP filters (Keyence). Digital images were processed using the BZ-II viewer application (Keyence) and Photoshop CS3 (Adobe).

Liposome co-sedimentation assay
Rabs (4 μM) were mixed with the Rh-labeled liposomes (1.5 mM lipids) in HN150 containing 6 mM MgCl2, and 1 mM DTT, and incubated (30°C, 2 hours). After centrifugation [50,000 rpm, 4°C, 30 min, TLA100 rotor (Beckman)], the pellets and supernatants were analyzed by SDS-PAGE.
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