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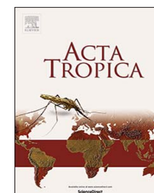

Proteasomal degradation of *T. gondii* ROP18 requires Derlin2

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ABSTRACT

T. gondii is an obligate intracellular parasite, belonging to the Phylum Apicomplexa, infecting all warm-blooded animals including humans. During host cell invasion, specialized cytoskeletal and secretory organelles play a pivotal role. ROP18, as a member of the ROP2 family, has been identified as a key virulence factor mediating pathogenesis in *T. gondii*. Here, we identify an ER-resident protein, Derlin2, a factor implicated in the removal of misfolded proteins from the ER for cytosolic degradation, as a component of the machinery required for ER-associated protein degradation (ERAD). We identified Derlin2 interacting with ROP18 by yeast two-hybrid screening system. The interaction between ROP18 and Derlin2 was further confirmed through *in vitro* GST pull-down and *in vivo* immunoprecipitation assays. By immunofluorescence assay, we found that ROP18 co-localized with Derlin2 in the endoplasmic reticulum. Using overexpression and knockdown approaches, we demonstrated that Derlin2 was required for *T. gondii* ROP18 degradation. Consistently, cycloheximide chase experiments showed that the degradation of ROP18 relied on the Derlin2, but not Derlin1. These results indicate that interaction between Derlin2 and ROP18 is functionally relevant and leads ultimately to degradation of ROP18. The finding provides the basis for future studies on Derlin2-dependent ERAD of *T. gondii* ROP18 and subsequent antigen generation.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite and an important opportunistic human pathogen, infecting a broad range of warm-blooded animals including humans, particularly in immunocompromised patients and developing foetuses (Moshfeghi et al., 2004; Sukthana, 2006). During host invasion, the contents of apical secretory organelles called rhoptries are discharged into the developing parasitophorous vacuole (PV) (Carruthers and Sibley, 1997; Dubremetz et al., 1993). In the process, some rhoptry proteins (ROPs) are also released directly into the host cell cytosol at the point of cell invasion. Among these effectors, ROP18 kinase, as a member of the ROP2 family, is located in the parasitophorous vacuole membrane (PVM) during invasion (Bradley et al., 2005). Endoplasmic reticulum-associated degradation (ERAD) is a protein clearance system that eliminates misfolded proteins or peptides from the endoplasmic reticulum (ER) to the cytosol (Smith et al., 2011), which includes three steps: recognition, dislocation and degradation (Lilley and Ploegh, 2004). Derlins are a family of membrane proteins involved in ERAD. Members of this family participate in the ER-associated degradation response, recognize substrates in the ER and retro-translocate them across the ER membrane into the cytosol. Derlins contain rhomboid pseudoprotease domains, which recognize and cleave protein sequences using hydrophilic residues across the membrane (Greenblatt et al., 2011). Derlin1 can form oligomers with Derlin2 and Derlin3 (Ye et al., 2005). Previous studies reported that the transmembrane domains of Derlin1 constitute a functional entity that is important for ERAD (Mehnert et al., 2014). However, Derlin2 has homology to yeast Der1p, which is similar to Derlin1 structure, and by linking to recognized target proteins results in proteasomal degradation in the cytosol (Ye et al., 2004). Reducing the levels of Derlin2 slows the degradation of a misfolded variant protein (Oda et al., 2006). Most recently, Derlin2 has been certified to play an essential role in HRD1-mediated retro-translocation of SHH and NHK (Huang et al., 2013). Derlin2 has also been shown to participate in

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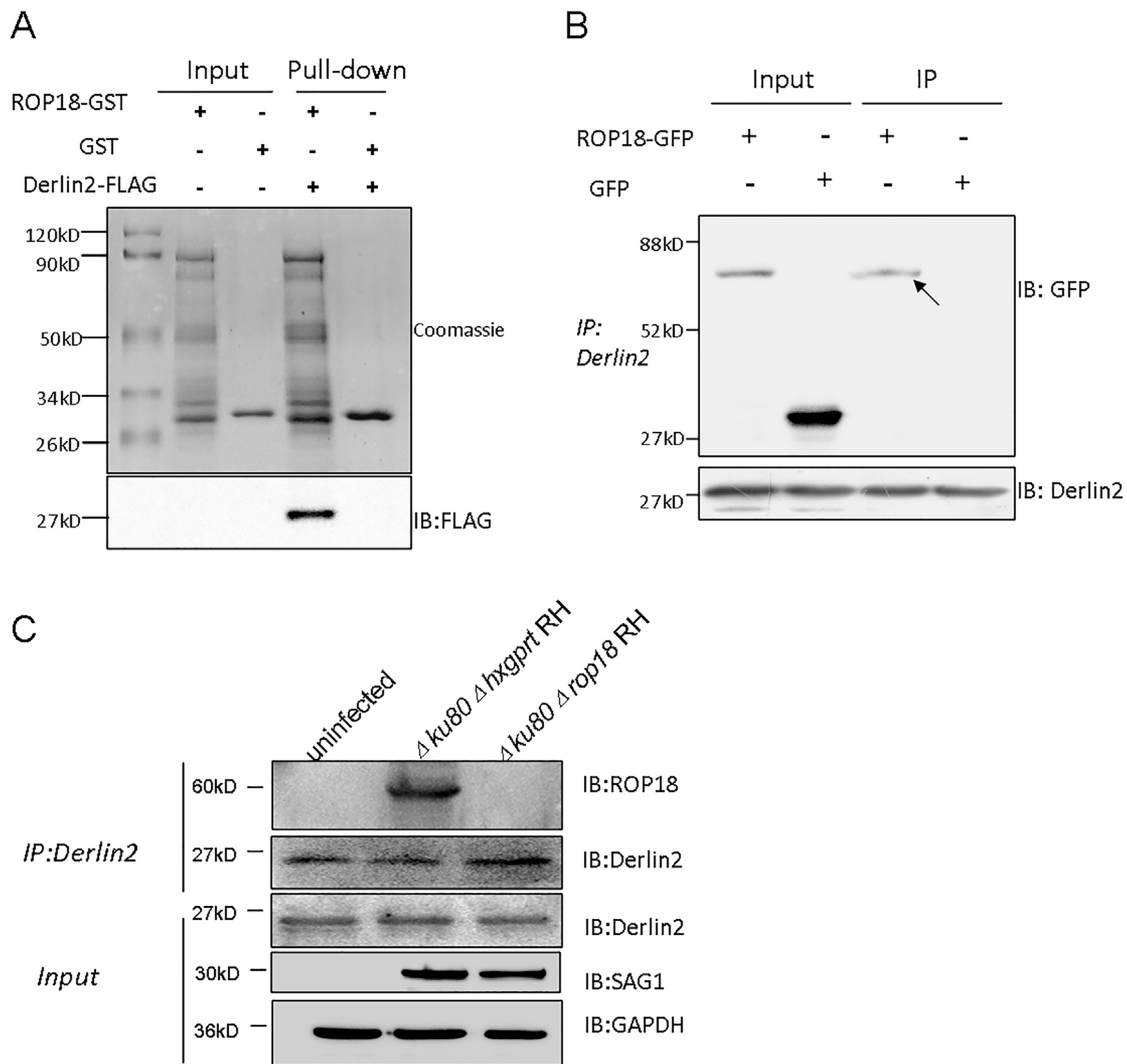

Fig. 1. Confirmation of the interaction of ROP18 with Derlin2 *in vivo* and *in vitro*.
(A) Confirmation of the Derlin2 bound to ROP18 using *in vitro* pull down assay. GST-ROP18 purified on glutathione beads was used as an affinity matrix for absorbing Derlin2. The SDS-PAGE gel was stained with Coomassie Brilliant Blue (upper panel) and subsequently blotted with DYKDDDDK Tag antibody (lower panel). (B) Verifying the interaction of ROP18 with Derlin2 through immunoprecipitation. HEK 293T cells were transfected with ROP18-GFP or control GFP vector. After 24 h transfection, the cell lysates were immunoprecipitated with Derlin2 antibody. Starting fractions (Input) and immunoprecipitates (IP) were detected by GFP and Derlin2 antibodies. The arrow indicates that ROP18 (upper panel) and Derlin2 (lower panel) were immunoprecipitated together. (C) Using ROP18 expressing transgenic strains to determine that ROP18 and Derlin2 interaction. The Neuro2a cells were infected with the indicated parasites at an m.o.i. of 1. At 12 h after infection, immunoprecipitation (IP) of Derlin2 from infected cell lysates was detected with rabbit polyclonal ROP18 antibody.

degradation of Proinsulin (Hoelen et al., 2015). So taken together this body of evidence shows that Derlin2, a constituent of ERAD complex, is involved in the dislocation of misfolded proteins from the ER into the cytosol (Lilley and Ploegh, 2005), which was required for ERAD (Oda et al., 2006).

The ROP18 kinase is as a key virulence factor conferring a high mortality phenotype of type I strains (Saeij et al., 2006). During the invasive stage, ROP18 is found in rhoptries and released into the PV (Smith et al., 2011). There is evidence that shows pathogen-driven host ER-PV interaction is an important route for antigen into the MHC class I pathway (Goldszmid et al., 2009). Simultaneously, numerous studies revealed various instances of pathogenic hijacking of the host ERAD pathway (Morito and Nagata, 2015). In this study, we report that ERAD of *T. gondii* ROP18 specifically requires Derlin2, but not Derlin1, for degradation. The finding provides the basis for future study of Derlin2-dependent ERAD of *T. gondii* ROP18 and subsequent antigen generation.

2. Materials and methods

2.1. Reagents and plasmids

Amplification of the full open reading frame encoding human Derlin2 (GenBank No. NM_016041.4) and Derlin1 (GenBank No. NM_001134671.2) were achieved through reverse transcriptase polymerase chain reaction of the whole mRNA of Hela cells. Amplification of the open reading frame encoding *T. gondii* ROP18 (GenBank ID: AM075204.1) was achieved through RT-PCR of the RH tachyzoite RNA. Then the full-length ROP18 was subcloned into the pGEX6P-1 vector (GE Healthcare, Wisconsin, USA) and pEGFP-C2 (BD Biosciences). The full length human Derlin2 and Derlin1 were subcloned into the 1xFLAG vector (Sigma, USA) and pCMV-3Tag-2A (BD Bioscience). All constructs were subject to sequencing for verification. Bip antibody (ab21685, Abcam, USA), Derlin1 antibody (ab176732, Abcam, USA), Derlin2 antibody (ab71552, Abcam, USA), GFP Rabbit antibody (2956s, CST,

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