ABCD_AZ756 and ABCD_AZ757 antibodies recognize Dictyostelium ubiquitinated proteins by western blot

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Abstract

Three antibodies (ABCD_AZ756, ABCD_AZ757 and ABCD_AZ758) were tested for their ability to recognize *Dictyostelium discoideum* ubiquitinated proteins by western blot. The results indicate that recombinant antibodies ABCD_AZ756 and ABCD_AZ757 successfully detect the accumulation of ubiquitinated proteins following treatment of cells with the proteasome inhibitor MG132.

Introduction

Proteasomes are large protein complexes responsible for the degradation of misfolded proteins in eukaryotic cells. Proteins destined for degradation by the proteasome are tagged with a chain of small molecules called ubiquitin. The peptide aldehyde MG132 is a widely used proteasome inhibitor (Lee & Goldberg, 1998). By blocking the proteasome activity, MG132 causes the accumulation of ubiquitinated proteins that can no longer be degraded. A previous study demonstrated that treating *D. discoideum* cells with the proteasome inhibitor MG132 results in the accumulation of ubiquitinated proteins, which appear as a smear on a western blot probed with a mouse monoclonal anti-ubiquitin antibody (BD Biosciences #550944; Santariaga *et al.*, 2018).

Antibodies ABCD_AZ756, ABCD_AZ757 and ABCD_AZ758 were developed by Hybrigenics SA and recognize different forms of ubiquitin. The antibody AZ756 was designed to recognize all ubiquitin forms (free, monoubiquitin as well as polyubiquitin chains) independently of the type of linkage. AZ757 and AZ758 were designed to recognize polyubiquitinated chains formed by lysine-48 (K48) and lysine-63 (K63) residue linkage, respectively.

Here, we describe the ability of AZ756 and AZ757 to detect the accumulation of ubiquitinated proteins in *D. discoideum* by western blot following treatment of the cells with the proteasome inhibitor MG132.

Materials & Methods

Antibodies: ABCD_AZ756, ABCD_AZ757 and ABCD_AZ758 antibodies (ABCD nomenclature, http://web.expasy.org/abcd/) were developed by Hybrigenics service SA. They were produced by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/) as nanobodies with the antigen-binding VHH portion fused to a rabbit IgG Fc. HEK293T suspension cells growing in HEK TF medium (Xell #861-0001, Sartorius), supplemented with 0.1% Pluronic F68 (Sigma-Aldrich, #P1300), were transiently transfected with the vector coding for the VHH-Fc of each antibody. Supernatants (AZ756: 190 µg/mL, AZ757: 220 µg/mL and AZ758: 210 µg/mL) were collected after 4 days.

Antigen: Wild-type *D. discoideum* cells (DH1-10) were treated with 20 µM or 100 µM of the proteasome inhibitor MG132 (Sigma-Aldrich, #474790) or DMSO (Sigma-Aldrich, #41640) as a negative control.

Protocol: $1x10^6$ cells were collected at the indicated time points after treatment with MG132. Cells were pelleted and lysed in 30 µL of sample buffer (20.6% (w/v) sucrose, 100 mM Tris pH 6.8, 10 mM EDTA, 0.1% (w/v) bromophenol blue, 4% (w/v) SDS) supplemented with 10mM dithiothreitol (DTT). Samples were then heated at 95°C for 5 minutes and 15 µL of each sample was migrated (200 V, 30 minutes) on a 4-15% acrylamide gel (SurePAGE Bis-Tris, Genscript #M00657) and transferred to a nitrocellulose membrane using a dry transfer system for 7 minutes (iBlot2 gel transfer device, Invitrogen #IB21001 and #IB23001). The membranes were blocked during 2 hours in PBS containing 0.1% (v/v) Tween20 and 6% (w/v) milk and washed three times for 5 minutes in PBS + 0.1% (v/v) Tween20. The membranes were then incubated with antibodies AZ756, AZ757 or AZ758 (1 µg/mL in PBS-Tween) overnight at 4°C. The next day, the membranes were washed three times for 5 minutes in PBS-Tween. The membranes were then incubated for 1 hour with horseradish peroxidase-coupled goat anti-rabbit IgG (Invitrogen #A16110, dilution 1:3000 in PBS-Tween) and washed three times for 5 minutes in PBS-Tween. The signal was revealed by enhanced chemiluminescence (ECL) (Millipore) using a PXi-4 gel imaging systems (Syngene).

Results & Discussion

To test the ability of AZ756, AZ757 and AZ758 to recognize ubiquitinated proteins by western blot, we used wild-type *D. discoideum* cell lysates treated with MG132 for 18 hours. Antibodies AZ756 and AZ757 detected accumulated high molecular weight species that most likely correspond to ubiquitinated proteins (Fig. 1A). The

intensity of the smears increased with higher MG132 concentrations, indicating a more pronounced accumulation of ubiquitinated proteins caused by the inhibitor. However, no clear signal was detected with AZ758 (Fig. 1A).

The absence of a detectable signal could be due to the limited sensitivity of the AZ758 antibody toward ubiquitinated proteins present in the samples. AZ758 is selective for K63-linked ubiquitin chains, which are primarily associated with proteasome-independent cellular pathways (Husnjak & Dikic, 2012). Consequently, their abundance is unlikely to be significantly affected by the proteasome inhibitor MG132. In contrast, K48-linked ubiquitin chains are the primary signals for targeting proteins to the proteasome for degradation.

AZ756 was then used to monitor the accumulation of ubiquitinated proteins over time (Fig. 1B). As expected, a progressive accumulation of ubiquitinated proteins was observed. Overall, these results demonstrate that the antibodies AZ756 and AZ757 successfully detect the accumulation of *D. discoideum* ubiquitinated proteins by western blot.

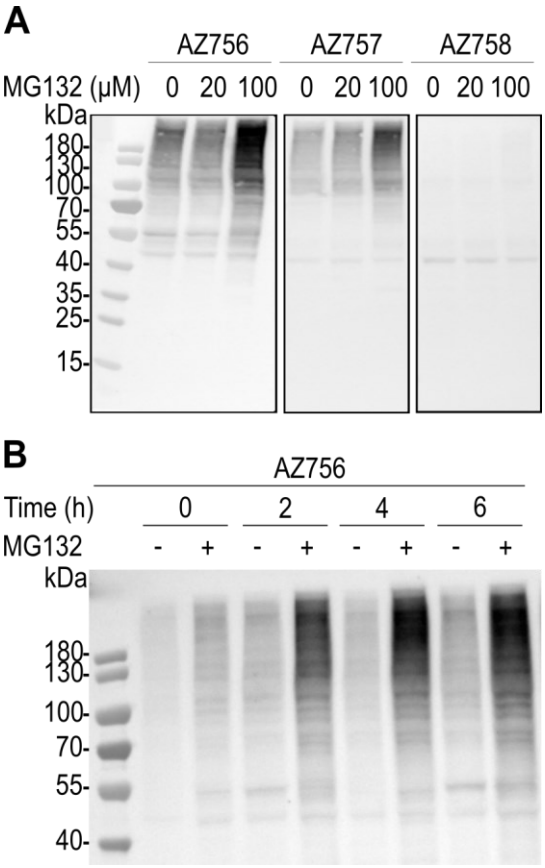

Fig. 1. (A) Specific binding of AZ756 and AZ757 to *D. discoideum* ubiquitinated proteins, as detected by western blot. No detection was observed with the antibody AZ758. Wild-type cells were treated with increasing concentrations of the proteasome inhibitor MG132 for 18 hours. **(B)** Western blot showing the kinetics of ubiquitinated protein accumulation using AZ756 antibody. Wild-type cells were treated with 100 μM MG132 and were collected at different time points.

References

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Conflict of interest

The author declares no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.