ABCD_RB877 and ABCD_RB878 antibodies recognize the Dictyostelium discoideum endogenous DstD protein by western blot

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Abstract

Four recombinant antibodies (ABCD_RB877 to ABCD_RB880) detect by ELISA a purified peptide from the *Dictyostelium discoideum* DstD protein. ABCD_RB877 and ABCD_RB878 also recognize the endogenous DstD and the GFP-DstD protein stably expressed in *D. discoideum* cells by western blot analyses.

Introduction

The *D. discoideum* DstD protein (or STATd, UniProt #Q86I20) belongs to the Signal Transducer and Activator of Transcription (STAT) protein family. DstD was identified in a computer search of the *Dictyostelium* genome combining BLAST data from the core motif sequence of the basic structure of SH2 with the whole SH2 domain (Gao *et al*., 2004). Here, we describe the ability of four recombinant antibodies (ABCD_RB876 to ABCD_RB880) to detect by ELISA a biotinylated peptide from the *D. discoideum* DstD protein. In addition, we determined that ABCD_RB877 and ABCD_RB878 antibodies were also able to recognize the endogenous DstD and the GFP-DstD protein stably expressed in *D. discoideum* by western blot analysis.

Materials & Methods

Antibodies: The antibodies ABCD_RB877, ABCD_RB878, ABCD_RB879 and ABCD_RB880 (ABCD nomenclature, http://web.expasy.org/abcd/, referred to collectively as RB877–880) were generated by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/). Briefly, a semi-synthetic human phage display library (Novimmune) was screened against a mix of two biotinylated peptides, DstD1 and DstD2, which correspond to amino acids 2–19 and 68–88, respectively, of the *D. discoideum* DstD protein (DstD1 sequence: SQREWTTASTSDLMSNLL; DstD2 sequence: QPLLRNASTNIFFGDDWTKQN). After three rounds of panning, selected phage vectors were isolated using a plasmid preparation kit (Qiagen), and the scFv inserts were subcloned into custom-made expression vectors and sequenced. The selected antibodies were expressed as mini-antibodies, consisting of the antigen-binding scFv domain fused to a human IgG1 Fc region. HEK293 suspension cells, cultured in HEK TF medium (Sartorius #861-0001) supplemented with 0.1% Pluronic F68 (Sigma #P1300), were transiently transfected with the vectors encoding the scFv-Fc constructs. Supernatants containing the mini-antibodies were harvested three days post-transfection. Estimated production yields were approximately 140-180 mg/mL for ABCD_RB877 and ABCD_RB880, and 75-110 mg/mL for ABCD_RB878 and ABCD_RB879.

Antigen: The same peptides used for phage selection were used as the antigens for ELISA detection.

ELISA: The whole procedure was carried out at room temperature. Biotinylated peptides at saturating concentration (10 pmol/well) were incubated on streptavidin-coated ELISA plates (Pierce #15124) for 30 min. Each well was rinsed three times with 100 µL of washing buffer (PBS + 0.5% (w/v) BSA + 0.05% (w/v) Tween20), then incubated for 1 hour with 50 µL of RB-mini-antibody-containing supernatant diluted in washing buffer (Fig. 1). After rinsing 3 times (100 µL of washing buffer), wells were incubated with horseradish peroxidase-coupled goat anti-human IgG (BioRad #1721050, dilution 1:1000, 50 µL per well) for 30 min. After 3 rinses, Tetramethylbenzidine (TMB) substrate (Sigma #T5569) was added (50 µL per well). The reaction was stopped by the addition of 25 µL of 2 M H_2SO_4. The absorbance (OD) was measured at 450 nm, and the absorbance at 570 nm was subtracted.

***D. discoideum* cell lines:** All cell lines were cultured in HL5c medium (HLH2, Formedium) and maintained at 22°C. The *dstD* knock-out (KO) cell line was generated in Ax2 (Ka) background (Bloomfield *et al*., 2018) using CRISPR/Cas9 technology. The pTM1285-guide1-dstD plasmid was used to express a guide RNA (gRNA) targeting the exon 1 of *dstD* (TGGACTAAACAGAATACAGT). Approximately 5.10^7 cells were detached with 1 mL cold H40 buffer (40 mM HEPES, 1 mM $MgCl_2$, pH 7) and washed twice with 1 mL cold H40 buffer. Then 5.10^6 cells were mixed with 2 µg of plasmid and electroporated in a 2 mm-gap cuvette by two pulses of 350 V and 8 msec duration separated by 1 sec interval. The cells were immediately diluted in 10 mL of HL5c medium and let to recover overnight. The transformants were selected with 10 µg/mL G418 (Sigma-Aldrich #A1720) and single cells were isolated on

Klebsiella pneumoniae KpGE strain agar plate (Lima *et al*., 2018). After clonal expansion, the populations were genotyped by DNA sequencing of the PCR-amplified genomic region targeted by the gRNA to confirm the desired edit. Ax2 (Ka) WT and *dstD*-KO cell lines expressing GFP-DstD were obtained using the "safe-haven" integrative pDM1513-GFP-DstD plasmid allowing an integration at the *act5* locus (Paschke *et al*., 2019). The cells were electroporated with 1 µg of linear DNA fragment, from the plasmid previously digested with NgoMIV (New England Biolabs #R0564S) as described in Paschke *et al*., using the electroporation protocol described above. The transformants were selected at a final concentration of 50 µg/mL of hygromycin (InvivoGen #ant-hg-5). After three days under selection, the transfected cells were gently washed and the fluorescence was verified by microscopy. Finally, the cells were FACS-sorted to select a homogenous population with intermediate fluorescence intensity.

Western blotting:

$5x10^6$ *D. discoideum* cells were collected from a 10 cm Petri dish, pelleted by centrifugation at 1,500 rpm and resuspended for washing with 1X Sorensen buffer (15 mM KH_2PO_4, 2mM Na_2HPO_4, 50 µM $MgCl_2$, 50 µM $CaCl_2$, pH 6.0), 120 mM Sorbitol, 1X cOmplete™ Inhibitor cocktail (Roche #05056489001) and 1X PhosSTOP™ (Roche #04906837001). After re-centrifugation, the cell pellet was lysed in 1X Laemmli loading buffer (62.5 mM Tris pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.005% (w/v) bromophenol blue, 5 mM DTT). Approximately $5x10^5$ cells corresponding to 25 µg of proteins were denatured 5 min at 95°C and loaded in wells of a 8% homemade polyacrylamide gel. Following migration, the proteins were wet transferred to a nitrocellulose membrane (Amersham Protran, catalog #GE10600002) in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS at 4 °C and 120 V for 1 hour and 30 minutes. For optimal detection in a first screening round, the membranes were blocked for 1 hour at room temperature in 1X TBS containing 5% (w/v) BSA (SERVA Electrophoresis GmbH #11930) and incubated overnight at 4°C with primary antibodies (RB877-880) at a 1:10 dilution in 1X TBS-Tween and 5% (w/v) BSA. In the second round with the positive antibodies, the membrane was blocked in 1X TBS-Tween and 3% (w/v) milk (GE Healthcare #RPN418) and incubated overnight at 4°C with primary antibodies diluted at 1:50 in the same buffer. The membranes were then washed three times for 5 minutes in 1X TBS-Tween and incubated for 1h at room temperature with goat anti-human IgG (H+L) coupled to horseradish peroxidase (BioRad #172-1050) in 1X TBS-Tween and 3% (w/v) milk. Following incubation, the membranes were washed three more times as described previously. The signal was revealed by enhanced chemiluminescence (ECL; Amersham Biosciences #RPN2232) using a Fusion Fx device (Vilbert Lourmat).

Results and discussion

The two DstD-derived peptides used in the phage display selection were evaluated for antibody binding by ELISA. As shown in Figure 1, all tested antibodies bound to the DstD2 peptide in a concentration-dependent manner, while no binding was detected with the DstD1 peptide. Given that antibody selection was performed using a mixture of both peptides, the absence of binding to DstD1 may be attributed to a lower antigenicity relative to DstD2.

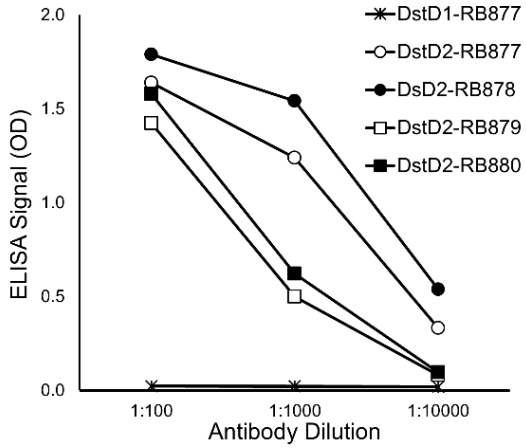

Fig. 1. Specific binding of ABCD_RB antibodies to the target *D. discoideum* DstD2-derived peptide but not to the DstD1-derived peptide (shown only for ABCD_RB877; other background curves are superimposed), as detected by ELISA.

The ability of the antibodies to bind the full-length protein was then assessed by western blot analyses (Fig. 2). Western blotting was performed using lysates from *D. discoideum* Ax2 (Ka). Lysates from *dstD*-KO cells were used as a negative control and lysates from wild type cells expressing the GFP-DstD protein were used as a positive control. In a first screen, blocking of the membrane was performed with 5% (w/v) BSA and primary antibodies were diluted 1:10. RB879 and RB880 recognized nonspecific background proteins. RB877 detected the GFP-DstD but failed to efficiently detect the endogenous DstD protein (Fig. 2). RB878 antibody recognized the endogenous DstD and the GFP-DstD proteins.

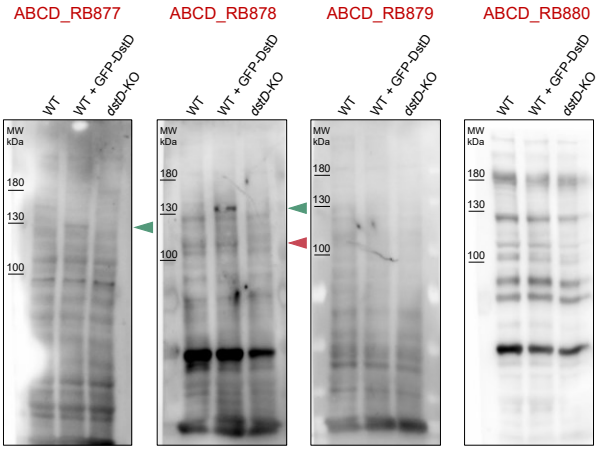

Fig. 2. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells expressing GFP-DstD and *dstD*-KO cells. Membranes were blocked with 5% (w/v) BSA for 1h and incubated overnight with the indicated ABCD_RB antibodies at a dilution of 1:10. Endogenous DstD has an expected size of ~93 kDa, and GFP-DstD of ~120 kDa. Green arrows indicate the GFP-DstD protein and the red arrow the endogenous DstD protein.

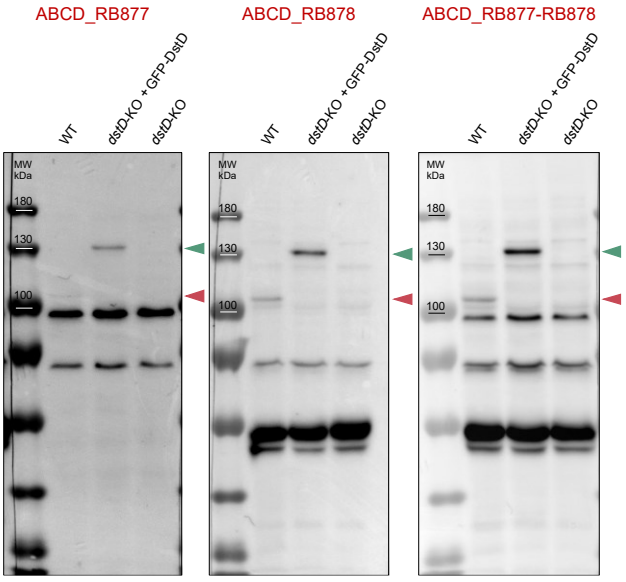

Fig. 3. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells, *dstD*-KO cells expressing GFP-DstD or *dstD*-KO cells. Membranes were blocked with 3% (w/v) milk for 1h and incubated with the indicated primary antibodies at a dilution of 1:50. Endogenous DstD has an expected size of ~ 93 kDa, and GFP-DstD of ~ 120 kDa. Green arrows indicate the GFP-DstD protein and red arrows the endogenous DstD protein.

The positive antibodies RB877 and RB878 were then tested again at a dilution of 1:50 following blocking of the membrane with 3% (w/v) milk (Fig. 3). In parallel, these two antibodies were also tested as an oligoclonal pool at a dilution of 1:50 each. For this assay, *dstD*-KO cells expressing GFP-DstD proteins were used as a positive control. Under these conditions, the RB877 antibody recognized the GFP-DstD protein and slightly detected the endogenous DstD. RB878 antibody recognized efficiently both proteins (Fig. 3). It is noteworthy that the mix of the two antibodies did not significantly improve the detection of the proteins and only increased the background noise. The results demonstrate that antibodies RB877 and RB878 specifically detect the DstD protein in both western blot and ELISA assays. In contrast, the other antibodies tested are ineffective in western blot but may be suitable for ELISA. These findings highlight RB877 and RB878 as reliable tools for the immunodetection of DstD.

References

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Conflict of interest

The authors declare 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.