Antibodies ABCD_RB861, ABCD_RB863 and ABCD_RB865 recognize the full-length DstA by western blot analysis

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Abstract

Ten recombinant antibodies (ABCD_RB859-868) detect by ELISA a peptide from the *Dictyostelium discoideum* DstA protein. Three of these antibodies, ABCD_RB861, ABCD_RB863 and ABCD_RB865, also recognize by western blot the endogenous DstA and the GFP-DstA protein stably expressed in *D. discoideum* cells.

Introduction

The *Dictyostelium discoideum* DstA protein (also known as STATa, UniProt #O00910) belongs to the Signal Transducer and Activator of Transcription (STAT) protein family (IPR001217). DstA was initially characterized as a TTGA-binding factor (Kawata *et al*., 1996; Kawata *et al.*, 1997) and shown to function as a transcriptional repressor that regulates stalk cell differentiation commitment (Mohanty *et al*., 1999). DstA also functions as an activator in the prestalk tip region (Fukuzawa and Williams, 2000; Saga *et al*., 2016). Its activity is modulated by tyrosine kinases (Araki and Williams, 2012; Araki *et al*., 2014; Saga *et al*., 2019) and phosphatases (Araki *et al*., 2008; Early *et al*., 2001). In this study, we describe the ability of ten recombinant antibodies (ABCD_RB859-868) to detect by ELISA a biotinylated peptide from the *D. discoideum* DstA protein. In addition, we show that the ABCD_RB861, ABCD_RB863 and ABCD_RB865 antibodies can also recognize endogenous DstA and the GFP-DstA protein stably expressed in *D. discoideum* by western blot analysis.

Materials & Methods

Antibodies: The antibodies ABCD_RB859, ABCD_RB860, ABCD_RB861, ABCD_RB862, ABCD_RB863, ABCD_RB864, ABCD_RB865, ABCD_RB866, ABCD_RB867, and ABCD_RB868 (ABCD nomenclature, http://web.expasy.org/abcd/, referred to collectively as RB859–868) were generated by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/). Briefly, a semi-synthetic phage display library (Novimmune) was panned against the N-terminally biotinylated peptide corresponding to amino acids 4 to 46 of the *D. discoideum* DstA protein (sequence: AEFSMDDFEDTFDSNATISTKDLFEGSDRLPLNQSI NTTIQNL). 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 (Xell #861-0001, Sartorius) 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 10–20 mg/L for antibodies RB859, RB860, RB862, RB863, and RB864, and 50–100 mg/L for the other antibodies.

Antigen: The same biotinylated peptide used for phage selection was used as the antigen for ELISA detection. An unrelated biotinylated peptide (sequence: GGMALDGIRMPDGSYADGT) was used as a negative control.

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 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 (Formedium #HLH2) and maintained at 22°C. The *dstA* knock-out (KO) cell line was generated in Ax2 (Ka) background (Bloomfield *et al*., 2018) using CRISPR/Cas9 technology. The pTM1285-guide1-dstA plasmid was used to express a guide RNA (gRNA) targeting the exon 2 of *dstA*

(TGATCACTTTGGTTTAATGG). Approximately 5.10^7 cells were detached with 1 mL cold H40 buffer (40 mM HEPES, 1 mM MgCl2, pH7) 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 (Lima *et al*., 2018) agar plate. 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 *dstA*-KO cell lines expressing GFP-DstA were obtained using the "safe-haven" integrative pDM1513-GFP-DstA 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*. 2019, 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 during 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 (RB859-RB868) at a 1:2 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

Antibodies RB859-868 bound the *D. discoideum* DstA peptide in a concentration-dependent manner but did not bind to the unrelated peptide used as a negative control (Fig. 1). The lower signals observed with antibodies RB860, RB862, and RB864 can probably be attributed to their lower concentrations.

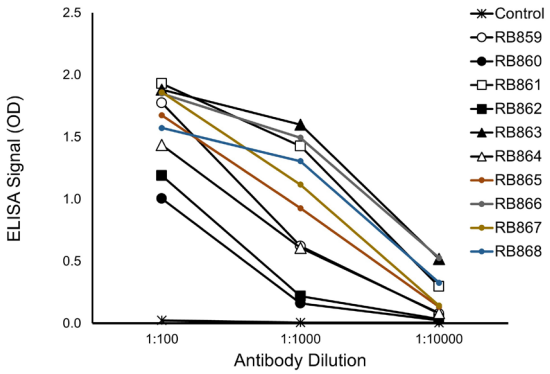

Fig. 1. Specific binding of ABCD_RB antibodies to the target *D. discoideum* DstA-derived peptide but not to the unrelated peptide used as a negative control (shown only for RB859; other background curves are superimposed), as detected by ELISA.

The ability of the RB859-868 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 *dstA*-KO cells were used as a negative control and lysates from wild type cells expressing the GFP-DstA 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 1:2. RB859, RB860, RB862, RB864, RB866, RB867 and RB868 only recognized nonspecific background proteins, while RB861, RB863 and RB865 antibodies recognized the endogenous DstA and the GFP-DstA proteins (Fig. 2).

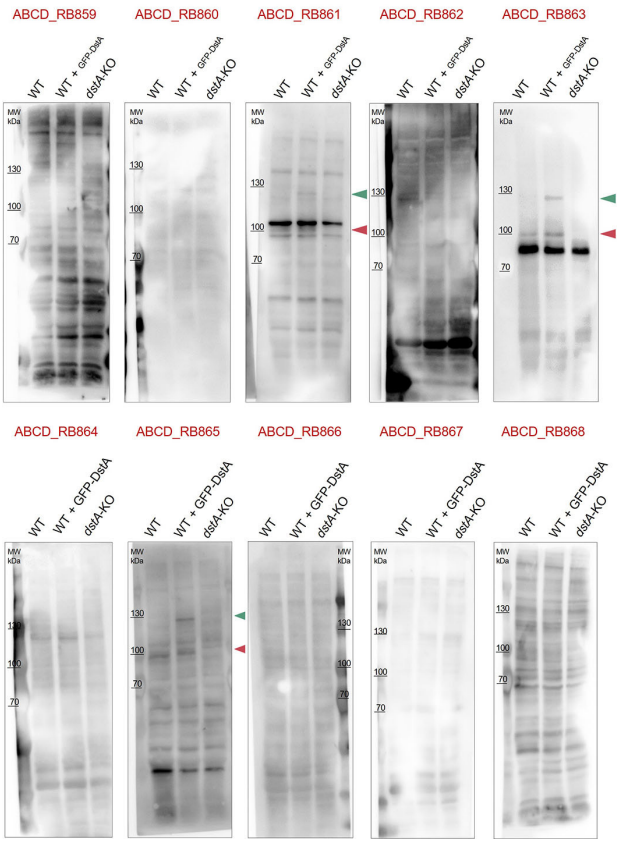

Fig. 2. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells, WT cells expressing GFP-DstA and *dstA*-KO cells. Membranes were blocked with 5% (w/v) BSA for 1h and incubated with the indicated ABCD_RB antibodies at a dilution of 1:2. Endogenous DstA has an expected size of ~ 80 kDa, and GFP-DstA of ~ 107 kDa. The green arrows indicate the GFP-DstA protein and the red arrows the endogenous DstA protein.

The positive antibodies RB861, RB863 and RB865 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 three antibodies were also tested as an oligoclonal pool at a dilution of 1:50 each. For this assay, *dstA*-KO cells expressing GFP-DstA proteins were used as a positive control. Under these conditions, the RB861 antibody did not significantly recognize the endogenous DstA and GFP-DstA proteins, whereas the RB865 antibody recognized efficiently both proteins (Fig. 3). Finally, the RB863 antibody recognized the endogenous DstA protein but failed to efficiently detect GFP-DstA protein due to the presence of non-specific bands. The mix of the three antibodies did not improve the detection of the proteins and only increased the background noise (Fig. 3).

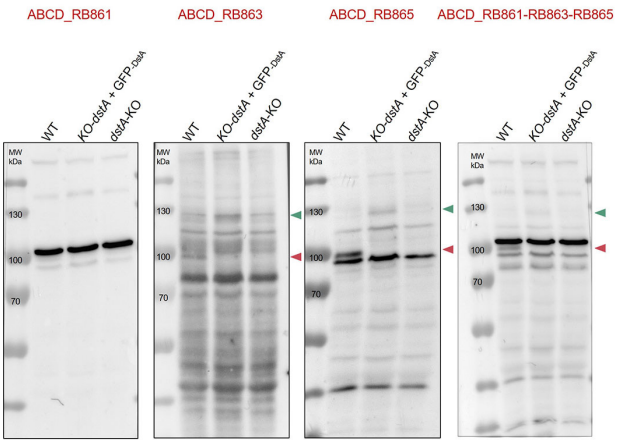

Fig. 3. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells, *dstA*-KO cells expressing GFP-DstA or *dstA*-KO cells. Membranes were blocked with 3% (w/v) milk for 1h and incubated with the indicated ABCD_RB antibodies at a dilution of 1:50. Endogenous DstA has an expected size of ~ 80 kDa, and GFP-DstA of ~ 107 kDa. The green arrows indicate the GFP-DstA protein and the red arrows the endogenous DstA protein.

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Conflict of interest

The authors declare no conflicts of interest.

Data Availability Statement

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