ABCD_RB874 antibody recognizes the endogenous DstC protein from Dictyostelium discoideum by western blot

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Abstract

Five recombinant antibodies ABCD_RB872, ABCD_RB873, ABCD_RB874, ABCD_RB875 and ABCD_RB876 detect by ELISA a purified peptide of the *Dictyostelium discoideum* DstC protein. ABCD_RB874 also recognizes the endogenous DstC and the GFP-DstC protein stably expressed in *D. discoideum* cells by western blot analyses.

Introduction

The *D. discoideum* DstC protein (or STATc, UniProt #Q54BD4) belongs to the Signal Transducer and Activator of Transcription (STAT) protein family. DstC was isolated through a low stringency hybridization screen using the SH2 domain of DstA as a probe (unpublished result from M. Fukuzawa, K. A. Jermyn, and J. G. Williams). DstC was shown to be activated by DIF, the inducer of prestalk cell differentiation, and functions as a transcriptional repressor (Fukuzawa *et al*., 2001; Fukuzawa *et al*., 2003). DstC is also activated in response to hyperosmotic stress, heat shock and oxidative stress to regulate transcriptional responses (Araki *et al*., 2003; Na *et al*., 2007). In addition to cGMP signaling triggered by hyperosmotic stress, intracellular Ca^{2+} is a parallel second messenger also involved in STATc regulation (Araki *et al*., 2010). The activity of DstC is regulated by tyrosines kinases (Araki *et al*., 2014; Lee *et al*., 2008) and phosphatases (Araki *et al*., 2008; Araki *et al*., 2010). Here, we describe the ability of five recombinant antibodies (ABCD_RB872-876) to detect by ELISA a biotinylated peptide from the *D. discoideum* DstC protein. In addition, we determined that ABCD_RB874 was also able to recognize the endogenous DstC and the GFP-DstC protein stably expressed in *D. discoideum* by western blot analyses.

Materials & Methods

Antibodies: Antibodies ABCD_RB872, ABCD_RB873, ABCD_RB874, ABCD_RB875 and ABCD_RB876 (ABCD nomenclature, http://web.expasy.org/abcd/, referred to collectively as RB872–876) 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 9 to 51 of the *D. discoideum* DstC protein (sequence: RPLDAISNTFEVKQEEPEFSSDGFNTTNDDLMSLMT FLDNGTG). After three rounds of panning, selected phage vectors were isolated using a plasmid preparation kit (Qiagen), and scFv inserts were subcloned into custom-made expression vectors and sequenced. Selected antibodies were then 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 160-180 mg/L for RB872, RB873 and RB874 and 100-110 mg/mL for RB875 and RB876.

Antigen: The same peptide used for phage selection was used as the antigen for ELISA detection. A peptide from the DstB protein (UniProt #Q70GP4) was used as a negative control (Sequence:IEKEQKLRSQCQAVEDIN AKLENENLQLKKELFEMSRKFKEIDII).

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 *dstC* knock-out (KO) cell line was generated in Ax2 (Ka) background (Bloomfield *et al*., 2018) using CRISPR/Cas9 technology. The pTM1285-guide1-dstC plasmid was used to express a guide RNA (gRNA) targeting the exon 2 of *dstC* (CAGCAACTCCACCAGCAATT). 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 left to recover overnight. Transfected cells 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 *dstC*-KO cell lines expressing GFP-DstC were obtained using the "safe-haven" integrative pDM1513-GFP-DstC 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. Transfected cells 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, 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, 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 (RB872-RB876) at a 1:10 dilution in 1X TBS-Tween and 5% (w/v) BSA. In the second round with the positive antibody, 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 membrane was 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 membrane was 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 & Discussion

Antibodies RB872, RB873, RB874, RB875 and RB876 bound the *D. discoideum* DstC peptide in a concentration-dependent manner but did not bind to the unrelated peptide from DstB used as a negative control (Fig. 1).

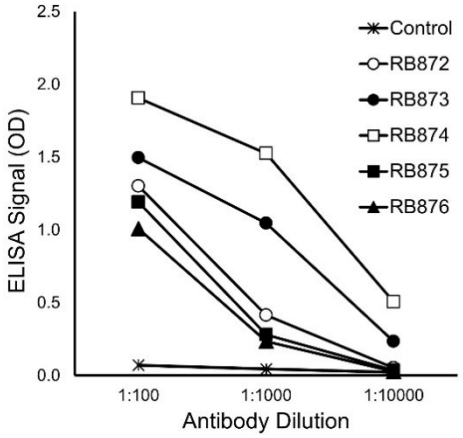

Fig. 1. Specific binding of ABCD_RB antibodies to the target *D. discoideum* DstC-derived peptide but not to the DstB peptide used as a negative control (shown only for ABCD_RB872; other background curves are superimposed), as detected by ELISA.

The ability of these antibodies to bind the full-length protein was assessed by western blot analyses (Fig. 2). Western blotting was performed using lysates from *D. discoideum* Ax2 (Ka). Lysates from *dstC*-KO cells were used as a negative control and lysates from wild type cells expressing the GFP-DstC protein were used as a positive control. In the first screen, blocking of membrane was performed with 5% (w/v) BSA and primary antibodies were diluted 1:10. RB872, RB873, RB875 and RB876 only recognized nonspecific background proteins while RB874 recognized the endogenous DstC and the GFP-DstC protein (Fig. 2). The higher apparent molecular

weight observed on the western blot compared to the expected size may be due to the N-terminal domain, mainly consisting of long runs of poly-asparagine and poly-glutamine residues, potentially affecting SDS binding and protein migration.

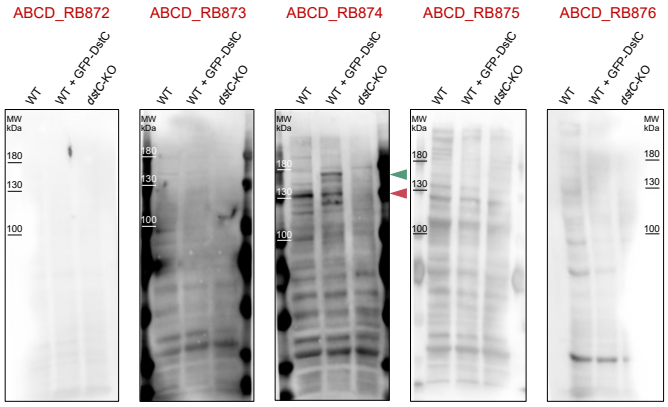

Fig. 2. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells expressing GFP-DstC and a *dstC*-KO cells. Membranes were blocked with 5% (w/v) BSA for 1h and incubated overnight with the indicated ABCD_RB antibodies at a dilution of 1:10. Endogenous DstC has an expected size of ~ 107 kDa, and GFP-DstC of ~ 134 kDa. The green arrow indicates the GFP-DstC protein and the red arrow the endogenous DstC protein.

The positive antibody RB874 was then tested again at a dilution of 1:50 following blocking of the membrane with 3% (w/v) milk (Fig. 3). For this assay, *dstC*-KO cells expressing GFP-DstC proteins were used as a positive control. Under these conditions, RB874 efficiently recognized the endogenous DstC and GFP-DstC proteins (Fig. 3). Overall, these results demonstrate that the antibody RB874 specifically detects DstC protein in both western blot and Elisa applications. In contrast, the other antibodies are not effective for western blot detection but might be used for ELISA. These findings confirm that the antibody RB874 is suitable for multiple immunodetection techniques, making it a valuable tool for protein analysis.

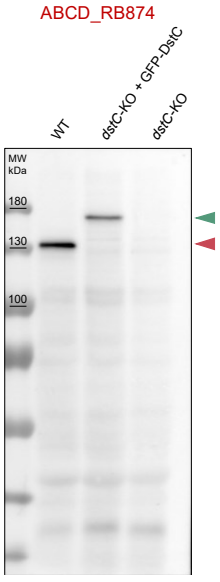

Fig. 3. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells, *dstC*-KO cells expressing GFP-DstC or *dstC*-KO cells. Membranes were blocked with 3% (w/v) milk for 1h and incubated with ABCD_RB874 antibody at a dilution of 1:50. Endogenous DstC has an expected size of ~ 107 kDa, and GFP-DstC of ~ 134 kDa. The green arrow indicates the GFP-DstC protein and the red arrow the endogenous DstC protein.

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Conflict of interest

Authors declare no conflict of interest.

Data Availability Statement

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

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