Antibodies ABCD_RB869-RB871 do not recognize the full-length DstB by western blot

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Abstract

Recombinant antibodies ABCD_RB869, ABCD_RB870 and ABCD_RB871 detect by ELISA a peptide from the *Dictyostelium discoideum* DstB protein. These antibodies do not recognize the endogenous DstB and a GFP-DstB protein stably expressed in *D. discoideum* cells by western blot analyses.

Introduction

The *D. discoideum* DstB protein (or STATb, UniProt #Q70GP4) belongs to the Signal Transducer and Activator of Transcription (STAT) protein family (IPR001217). DstB was identified through a low-stringency hybridization screen using the SH2 domain of DstA as a probe and was found to possess a divergent SH2 domain compared to those of STAT proteins in metazoans. Microarray analysis revealed differential gene expression regulation in *dstB*-null cells, indicating that DstB is biologically functional. Furthermore, like other STAT proteins, DstB was observed to undergo dimerization and nuclear enrichment upon activation (Zhukovskaya *et al*., 2004). Here, we describe the ability of three recombinant antibodies (ABCD_RB869-RB871) to detect by ELISA a biotinylated peptide from the *D. discoideum* DstB protein. In addition, we determined that these three antibodies were not able to recognize the endogenous DstB protein as well as a GFP-DstB protein stably expressed in *D. discoideum* by western blot analyses.

Materials & Methods

Antigen selection: A short coding sequence in the N-terminal domain of the *D. discoideum* DstB, corresponding to amino acids 220 to 264 (sequence: IEKEQKLRSQCQAVEDINAKLENENLQLKKELFEM SRKFKEIDII), was selected as antigenic peptide. The selection was based on the predicted structure of the protein. A BLAST analysis against the *D. discoideum* proteome confirmed the specificity of this protein region.

Antibodies: The antibodies ABCD_RB869, ABCD_RB870, and ABCD_RB871 (ABCD nomenclature, http://web.expasy.org/abcd/, referred to collectively as RB869–871) were generated by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/). Briefly, a semi-synthetic phage display library (Novimmune) was panned against the biotinylated DstB peptide. 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 130-180 mg/L for the three antibodies.

ELISA Antigen: The same biotinylated peptide used for phage selection was used as the antigen for ELISA detection. A biotinylated peptide from the DstC protein (UniProt #Q54BD4) was used as a negative control (Sequence: RPLDAISNTFEVKQEEPEFSSDGFNTTNDDLMSLMT FLDNGTG).

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 ABCD_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 *dstB* knock-out (KO) cell line was generated in Ax2 (Ka) background (Bloomfield *et al*., 2008) using CRISPR/Cas9 technology. The pTM1285-guide2-dstB plasmid was used to express a guide RNA (gRNA) targeting the exon 1 of *dstB* (TATCGGGTATAGATGATGAT). 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 (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) cell line expressing GFP-DstB was obtained using the "safe-haven" integrative pDM1513-GFP-DstB 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 in 1X Sorensen buffer (15 mM KH_2PO_4, 2mM Na_2HPO_4, 50 µM $MgCl_2$, 50 µM $CaCl_2$, pH 6.0) additioned with 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 an 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% MeOH, 0.01% SDS at 4 °C, 120 V, 1h30. The membranes were then 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 the primary antibodies at 1:2 dilution in 1X TBS-Tween and 5% (w/v) BSA. Following incubation, the membranes were washed three times for 5 minutes in 1X TBS-Tween, incubated 1 h at room temperature with goat anti-human IgG (H+L) coupled to horseradish peroxidase (BioRad #172-1050) in 1X TBS-Tween and 5% (w/v) BSA and then washed three times as previously. The signal was revealed by enhanced chemiluminescence (ECL) (Amersham Biosciences #RPN2232) using a Fusion Fx device (Vilbert Lourmat).

Results and discussion

Antibodies RB869, RB870 and RB871 bound the *D. discoideum* DstB peptide in a concentration-dependent manner but did not bind to the unrelated peptide from DstC used as negative control (Fig. 1).

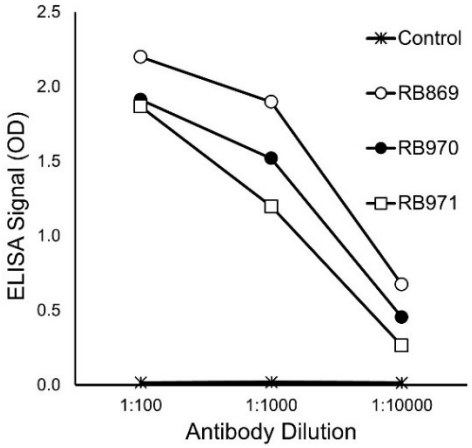

Fig. 1. Specific binding of ABCD_RB antibodies to the target *D. discoideum* DstB-derived peptide but not to the DstC peptide used as a negative control (shown only for RB869; other background curves are superimposed), as detected by ELISA.

The ability of all three antibodies to bind the full-length protein was assessed by western blot analyses (Fig. 2). The antibodies were tested on lysates from *D. discoideum* Ax2 (Ka) cells, with *dstB*-KO cells serving as a negative control. Additionally, lysates from wild-type cells expressing the GFP-DstB protein were used as a positive control. The three antibodies only recognized nonspecific background proteins and did not detect the endogenous DstB and the GFP-DstB proteins (Fig. 2). The lack of specific DstB recognition may be attributed to either the low affinity of the antibodies for the antigen or the low expression levels of DstB under standard growth conditions. Additionally, structural differences between the synthetic peptide and the native protein in its cellular context may contribute to the absence of specific binding. Overall, these results indicate that while the three antibodies are ineffective for detecting the DstB protein by western blot, they may still be suitable for use in ELISA-based assays.

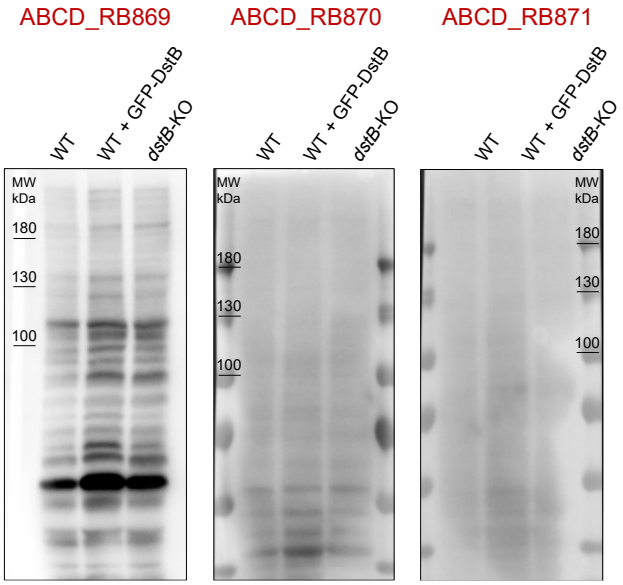

Fig. 2. Western blot on lysates from wild type *D. discoideum* Ax2 (Ka) cells, cells expressing GFP-DstB and a *dstB*-KO cell line. Membranes were blocked with 5% (w/v) BSA for 1h and incubated with the indicated ABCD_RB antibodies at a dilution of 1:2 overnight. Endogenous DstB has an expected size of ~ 129 kDa, and GFP-DstB of ~ 156 kDa.

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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.