The AL626 antibody recognizes an ALFA-tagged recombinant protein by immuno-electron microscopy

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Abstract
The AL626 antibody against the ALFA tag recognizes an ALFA-tagged human CD1b protein by immuno-electron microscopy in transfected HEK293T cells.

Introduction
The ALFA tag is a rationally designed epitope tag, forming a small (15 aa), stable α-helix, uncharged at physiological pH and resistant to chemical fixation. A nanobody (NbALFA) specifically recognizes this tag in native or fixed conditions. The NbALFA nanobody is thus suitable for a wide range of applications, including super-resolution microscopy, immunoprecipitation, western blotting, and electron microscopy (Götzke *et al.*, 2019). Here, we show that the AL626 recombinant antibody, derived from NbALFA, detects an ALFA-tagged human CD1b protein (UniProt P29016) by cryo-immuno electron microscopy in HEK293T cells.

Materials & Methods
Antibodies: The ABCD_AL626 antibody (ABCD nomenclature, https://web.expasy.org/abcd/) was produced by the Geneva Antibody Facility (https://www.unige.ch/medecine/antibodies/) as a mini-antibody with the antigen-binding VHH fused to a rabbit IgG Fc (for immunofluorescence) or to a mouse IgG2A Fc (for cryo-immunogold labelling). The synthesized VHH sequence (GeneArt, Invitrogen) corresponds to the variable region of the nanobody NbALFA (Götzke *et al.*, 2019). HEK293 suspension cells (growing in FreeStyle™ 293 Expression Medium, Gibco 12338) were transiently transfected with the vector coding for the VHH-Fc. Supernatant (100 mg/L) was collected after 4 days.

Antigen: HEK293T cells (growing in DMEM GlutaMAX™ [Gibco 31966]; supplemented with 10% Fetal Bovine Serum [Gibco 10270] and 100 µg/mL of penicillin-streptomycin [Gibco 15070-063]) were cultured at 37 °C, 5% CO_2 on glass coverslips (Menzel-Gläser, 22x22 mm) in 6-well tissue culture plates (Falcon 353046) for immunofluorescence as previously described (Lima and Cosson, 2020). Cells were grown in 100 mm tissue culture dishes (Falcon 353003) for immuno-electron microscopy. Cells transiently transfected 3 days before the experiment with a plasmid expressing an ALFA-tagged CD1b protein were used to detect the peptide tag. The ALFA epitope (`PSRLEEELRRRLTE`) was inserted in the C-terminal cytosolic domain of the fusion protein. The plasmid expressing a CD1b chimeric protein with a transmembrane domain composed of 21 hydrophobic residues was described previously (Mercanti *et al.*, 2010).

Protocol:
For *immunofluorescence*, the whole procedure was carried out at room temperature: transfected HEK293T cells were fixed with PBS containing 4% (w/v) paraformaldehyde (Applichem A3813) for 30 min, and blocked with PBS supplemented with 40 mM ammonium chloride (NH_4Cl) (Applichem A3661). Cells were then permeabilized in PBS containing 0.2% (w/v) saponin (Sigma S7900) for 3 min, washed once (3 min) with PBS supplemented with 0.2% (w/v) BSA (PBS-BSA), and incubated for 30 min with rabbit AL626 (final concentration 2 mg/L). After 3 washes (3 min) with PBS-BSA, cells were incubated for 30 min in PBS-BSA with secondary goat anti-rabbit IgG conjugated to AlexaFluor-647 (1:400, Thermo Fisher A21245). After 3 washes (3 min) with PBS-BSA and 1 wash with PBS (3 min), cells were mounted on slides (Menzel-Gläser, 76x26 mm) with Möwiol (Hoechst) containing 2.5% (w/v) DABCO (Fluka 33480). Pictures were taken using a Zeiss LSM800 Airyscan confocal microscope (Bioimaging Core Facility, Faculty of Medicine, University of Geneva), with a Plan-Apochromat 63x/1.40 Oil DIC f/ELYRA oil immersion objective.

For *immuno-electron microscopy*, cells were prepared following the Tokuyasu method (Tokuyasu *et al.*, 1978, Peters *et al.*, 2006): transfected HEK293T cells were fixed 15 min by adding 10 mL of 0.1 M pH 6.9 PHEM buffer (for 100 mL of 0.2 M pH 6.9 PHEM buffer: 120 mM PIPES [Sigma 80635]; 50 mM HEPES [Applichem A3724]; 4 mM Magnesium chloride hexahydrate [Sigma M2670]; 20 mM EGTA [Sigma E4378]; 1 M Sodium hydroxide [Applichem A3910] for pH adjustment) containing 4% (w/v) paraformaldehyde and 0.4% (w/v) glutaraldehyde (Sigma G7651) directly to the 10 mL of culture medium. The fixative was removed and HEK293T cells were then fixed further in 10 mL 0.1 M pH 6.9 PHEM buffer containing 2% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde for 2 hours. Cells were rinsed 5 times (2 min) with 0.1 M pH 6.9 sodium phosphate buffer (PB: 38 mM Sodium phosphate monobasic monohydrate [Sigma 59638]; 162 mM Sodium phosphate dibasic dihydrate [Sima 71643]), scraped in 1 mL of preheated 1% (w/v) gelatin in 0.1 M PHEM pH 6.9 buffer (Twee Torens Delft) and transferred into a microcentrifuge tube. Cells were centrifuged 3 min at 150 g, the supernatant was discarded and 1 mL of preheated 12% (w/v) gelatin in 0.1 M PHEM pH 6.9 was added by pipetting up and down twice quickly. The tube was incubated 5 min at 37 °C, mixed by pipetting up and down twice, then centrifuged 3 min at 950 g. The supernatant was discarded, and the pellet

hardened at 4 °C for 15 min. Then, the gelatin embedded cells were cut at 4 °C into 0.5 mm^3 cubes with a razor blade. Cells were cryoprotected by infiltration of 0.1 M PB containing 2.3 M sucrose (Sigma 84100) for at least 4 h at 4 °C on a rotating wheel. Gelatin blocks were placed onto aluminum specimen holders with sucrose and plunged into liquid nitrogen. Specimens were cryosectioned into 50 nm-thick slices using a Leica EM FCS ultramicrotome (Pôle Facultaire de Microscopie Électronique, Faculty of Medicine, University of Geneva) at -120 °C using a Diatome Cryo P 35° angle diamond knife at a speed of 1 mm/s. Sections were picked up with a mixture of 2% (w/v) methylcellulose 25 cps (Sigma M6385) and 2.3 M sucrose solution (1:4 ratio) using a stainless steel loop, and deposited onto the shiny side of a 200 mesh Formvar-coated copper grid (EMS FF200H-CU-50). Grids with sections were placed upside down onto PBS at 40 °C for 1 h, the section side of the grid was rinsed once on a drop of warm PBS, then successively on 5 drops (2 min each) of PBS containing 20 mM glycine (Applichem A1067) at room temperature. The grid was incubated 15 min on a drop of PBS supplemented with 1% (w/v) BSA, then 1 h on an 8 µL drop of mouse AL626 in PBS supplemented with 1% (w/v) BSA (final concentration 2 mg/L). The grid was rinsed on 5 drops (2 min each) of PBS containing 0.1% (w/v) BSA, then incubated 20 min onto an 8 µL drop of goat anti-mouse IgG conjugated to 10 nm gold particles (1:5 in PBS supplemented with 1% (w/v) BSA [BBi Solutions EM.GAM10]). The grid was rinsed successively on 6 drops (2 min each) of PBS containing 0.1% (w/v) BSA and on 6 drops (2 min each) of PBS. Samples were fixed on a drop of 1% (w/v) glutaraldehyde in PBS for 5 min and rinsed successively on 10 drops (1 min each) of ultrapure water. The grids were then stained on 1 drop of 2% (w/v) uranyloxalate pH 7 (2% (w/v) Uranyl acetate dihydrate [Fluka 94260]; 1.9% (w/v) oxalic acid dihydrate [Fluka 75700]; ammonium hydroxide [Sigma 105432] for pH adjustment), rinsed on 2 drops of ultrapure water, then rinsed on 2 drops of methylcellulose supplemented with 0.4% (w/v) uranylacetate pH 4 at 4 °C and incubated on a drop of methylcellulose supplemented with 0.4% (w/v) uranylacetate pH 4 for 5 min at 4 °C. Grids were picked up with a metal loop, excess solution absorbed by filter paper and grids were left to dry at room temperature for 15 min. Pictures were taken using a FEI Morgagni transmission electron microscope (Pôle Facultaire de Microscopie Électronique, Faculty of Medicine, University of Geneva).

Results

As previously reported (Lima and Cosson, 2020), the AL626 antibody specifically recognized the ALFA-tagged CD1b protein by immunofluorescence (Fig. 1A, left and middle panels). A specific signal was detected at the plasma membrane and in an intracellular compartment reminiscent of the ER network. No signal was detected in non-transfected cells (Fig. 1A, right panel). The AL626 antibody also specifically detected by immuno-electron microscopy a signal at the plasma membrane (Fig 1B, arrowhead) and at the level of the ER membrane (Fig 1B, arrows) in cells expressing ALFA-tagged CD1b.

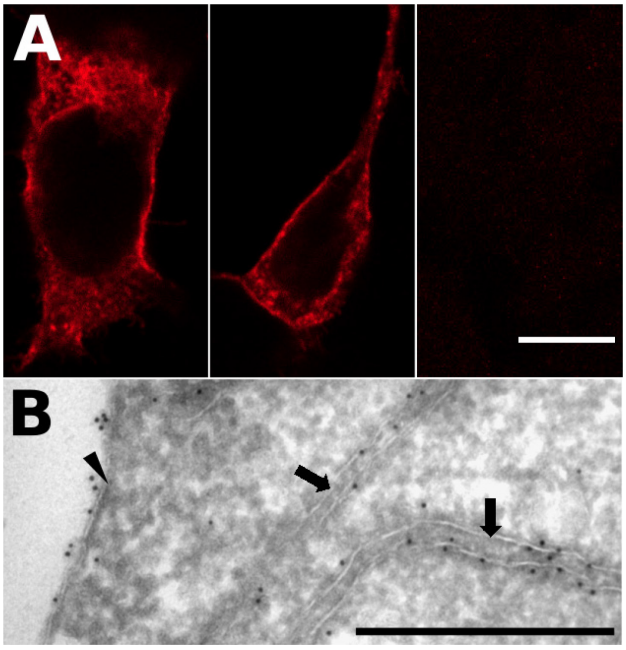

Fig. 1. (A) AL626 labelled the plasma membrane and ER of HEK293T cells expressing the ALFA-tagged CD1b protein (left and middle panel) by immunofluorescence. Non-transfected cells showed no signal (right panel). Scale bar: 10 µm. **(B)** An electron micrograph of cryo-immunogold labelled HEK293T cells expressing an ALFA-tagged CD1b protein presented a specific signal at the plasma membrane (arrowhead) and in the ER (arrows). Scale bar: 500 nm.

References

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Conflict of interest

Pierre Cosson is an editor of the Antibody Reports journal.