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Endosomal acidic pH-induced conformational changes of a cytosol-penetrating antibody mediate endosomal escape

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ABSTRACT

Endosomal escape after endocytosis is a critical step for protein-based agents to exhibit their effects in the cytosol of cells. However, antibodies internalized into cells by endocytosis cannot reach the cytosol due to their inability to escape from endosomes. Here, we report a unique endosomal escape mechanism of the IgG-format TMab4 antibody, which can reach the cytosol of living cells after internalization. Dissociation of TMab4 from its cell surface receptor heparan sulfate proteoglycan by activated heparanase in acidified early endosomes and then local structural changes of the endosomal escape motif of TMab4 in response to the acidified endosomal pH were critical for the formation of membrane pores through which TMab4 escaped into the cytosol. Identification of structural determinants of endosomal escape led us to generate a TMab4 variant with ~3-fold improved endosomal escape efficiency. Our finding of the endosomal escape mechanism of the cytosol-penetrating antibody and its improvement will establish a platform technology that enables a full-length IgG antibody to directly target cytosolic proteins.

1. Introduction

The ability of an antibody to reach the cytosol of target mammalian cells from outside of cells is highly desired for diverse purposes of research, diagnostic and therapeutic applications [1–3]. However, like other proteins and peptides, antibodies cannot passively diffuse across the plasma membrane of living cells owing to their large size and hydrophilicity [2,3]. Instead, they can be internalized into cells *via* receptor-mediated endocytosis after binding to cell surface receptors. After internalization, the antibody-receptor cargo is first transported to early endosomes (EEs), which serve as sorting stations for the internalized cargo, and subsequently routes to late endosomes (LEs) and finally lysosomes for degradation or can be transported back out of cells by recycling endosomes through the receptor- or neonatal Fc receptor-mediated recycling pathway [4,5]. To reach the cytosol, antibodies need to escape from endosomes along the endocytic pathway; however, they cannot do due to the inability of endosomal escape. Furthermore, the tight association of antibodies with the antigen receptor hampers their recycling or endosomal escape, causing lysosomal degradation, as exploited in antibody-drug conjugate technology [4]. To overcome these limitations of antibody itself, antibody delivery systems into the cytosol of living cells, such as polymeric micelles [6] and cell-penetrating and fusogenic peptide-embedded liposomes [7], have been recently developed.

Some bacterial toxins and cell-penetrating peptides (CPPs) that are derived from natural proteins or have been designed have the ability to reach the cytosol of cells after endocytosis from outside of cells [2,3]. Although the detailed endosomal escape mechanisms of most toxins and CPPs are still poorly defined, they are believed to exploit changes in the endosomal environment, such as an acidified pH, a reducing environment and/or activation of some proteases, to trigger endosomal escape during endosomal trafficking [2,8,9]. Importantly, the pH of the endocytic compartments progressively decreases from EEs (pH 5.5–6.5) to LEs (pH 4.5–5.5) to lysosomes (pH < 5.0) by the vesicular proton-ATPase pump [2,5]. The acidified endosomal pH plays a critical role to make toxins and CPPs undergo the necessary secondary/tertiary structural changes for interactions with the phospholipids of endosomal membranes to cause endosomal escape by membrane pore formation [8,9]. However, their endosomal escape efficiency is extremely low (less than 4% of the internalized molecule pools), with a negligible fraction of the endocytosed cargo reaching the cytosol [10,11], which limits their uses for the cytosolic delivery of biologically active cargos [12–15].

We recently reported the cytosol-penetrating antibody TMab4, a so-called cytotransmab, which in the intact human IgG1 format can access the cytosol of living cells after internalization through clathrin-mediated endocytosis using cell surface-expressed heparan sulfate

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proteoglycan (HSPG) as a receptor [16,17]. However, the endosomal escape mechanism has not been elucidated. Here, we report that TMab4 undergoes conformational changes in response to the acidified endosomal pH in order to cause endosomal membrane pore formation for the endosomal escape of TMab4. Through systematic mutational and functional studies, we identified the structural determinants of TMab4 that respond to the acidic pH for conformational changes of the endosomal escape motif to induce interaction with endosomal membranes, resulting in membrane pore formation. Understanding the endosomal escape mechanism allowed us to generate a TMab4 variant with much improved endosomal release efficiency, thereby efficiently reaching the cytosol from outside of cells.

2. Materials and methods

2.1. Construction of TMab4 variant expression plasmids

The mammalian expression plasmid, pcDNA 3.4-LC, encoding the light chain (LC) of TMab4 with hT4 light chain variable domain (VL) and Cκ constant domain sequence (residues 108-214 in EU numbering) was described before [16]. Site-directed mutagenesis for generating hT4 VL variants of TMab4 variants was performed by overlapping PCR using designed oligonucleotides (Macrogen Inc., Korea) [18]. The hT4-03 was prepared by DNA synthesis (Bioneer Inc., Korea). The mutated hT4 variants were subcloned into the *Not*I/*Bsi*WI site of the pcDNA 3.4-LC vector for LC expression [16]. All of the constructs were confirmed by sequencing (Macrogen Inc.).

2.2. Expression and purification of IgG antibodies

The mammalian expression plasmid, pcDNA 3.4-HC, encoding the heavy chain (HC) of TMab4 with heavy chain variable domain (VH) and human IgG1 constant domain sequence (CH1-hinge-CH2-CH3, residues 118–447 in EU numbering) was described before [16]. The plasmids that encode HC and LC of TMab4 and its variants were transiently co-transfected in pairs at an equivalent molar ratio into 0.2–1 L of HEK293F cell cultures in Freestyle 293F media (Invitrogen) following a previously described standard protocol [17,19,20]. Culture supernatants were harvested after 7 d culture at 37 °C in a humidified 8% CO_2 incubator by centrifugation and filtration (pore size 0.22 μm, cellulose acetate, Corning, CLS430521). Antibodies were purified from the culture supernatants using a protein-A agarose chromatography column (GE Healthcare) and dialyzed to achieve a final buffer composition of PBS with pH 7.4. Prior to cell treatments, antibodies were sterilized by filtration using a cellulose acetate membrane filter (pore size 0.22 μm, Corning). Antibody concentrations were determined both using a Bicinchoninic Acid (BCA) Kit (Pierce, 23225) and by measuring absorbance at 280 nm.

2.3. Modeling of TMab4 variable fragment (Fv)

Modeling of the three-dimensional structure of TMab4 Fv from the primary amino acid sequence was performed on the web antibody modeling (WAM) algorithm [21]. The WAM algorithm performs homology modeling of VH and VL frameworks (FRs) and complementarity-determining regions (CDRs) by aligning the submitted sequence to the most sequence-homologous FRs and CDRs of the same canonical class, respectively, from the Brookhaven Protein Data Bank of known Ab structures. The modeling was visualized with COOT program [22].

2.4. Confocal immunofluorescence microscopy

Confocal microscopy was performed for detection of internalized cytotransmabs or FITC-TAT in cultured cells, as described before [20,23,24]. Briefly, cells (2×10^4) grown on 12-mm diameter coverslips in 24-well culture plates were treated with indicated antibodies or FITC-TAT, as specified in the figure legends. To determine effects of pharmacological inhibitors, HeLa cells were pretreated with wortmannin (0.2 μM), bafilomycin (0.2 μM), and brefeldin A (7 μM) for 30 min and then treated with TMab4 or FITC-TAT. After 2× washes with PBS, the cells were washed 2× for 30 s at 25 °C with low-pH glycine buffer (200 mM glycine, 150 mM NaCl, pH 2.5), followed by 2 additional washes with PBS to remove non-internalized and nonspecifically surface-bound antibodies [23,24]. After fixation with 4% paraformaldehyde (PFA) in PBS for 10 min at 25 °C, permeabilization with PERM-buffer (0.1% saponin, 0.1% sodium azide, 1% BSA in PBS) for 10 min at 25 °C, and then blocking with 2% BSA in PBS for 1 h at 25 °C, the internalized cytotransmab was detected with Alexa488- or Alexa555-conjugated goat anti-human IgG antibodies for 2 h at 25 °C. Nucleus was stained with Hoechst 33342 for 5 min in PBS. After mounting the coverslips onto glass slides with Perma Fluor aqueous mounting medium (Thermo Scientific, TA-030-FM), center-focused single z-section images were obtained on a Zeiss LSM710 system with ZEN software (Carl Zeiss). In case of using 63× objective lens in confocal microscope, zoom factor 2 was applied for better resolution. The FITC or Alexa488 fluorescence was quantified using ImageJ software (NIH, USA) [1].

2.5. Pulse-chase experiments to monitor intracellular trafficking of TMab4

HeLa cells (2×10^4) grown on coverslips in 24-well culture plates were incubated with TMab4 (3 μM) or FITC-TAT (20 μM) for 30 min, quickly washed 3× with PBS, and then incubated at 37 °C for the indicated periods. After washing with PBS, stripping with low pH glycine buffer, fixation, permeabilization, and blocking of the cells, the internalized TMab4 were stained with Alexa488-conjugated goat anti-human antibody, and subcellular endocytotic organelles were stained with anti-Rab5 and anti-Flag for Flag-Rab11, followed by the appropriate FITC- or TRITC-conjugated secondary antibodies. The plasmid encoding Flag-Rab11 was transiently transfected 24 h before the treatment of TMab4. Lysosomes were visualized with LysoTracker® Red DND-99 that was diluted in medium (1 μM) and pretreated for 30 min at 37 °C before the cellular fixation. The subsequent confocal microscopy was performed as described above.

To examine effects of heparanase knockdown, control siRNA- and heparanase siRNA-treated HeLa cells were incubated with TMab4 or TAT for 30 min, quickly washed 3× with PBS, and then incubated at 37 °C for 2 h. After washing with PBS, stripping with low pH glycine buffer, fixation, permeabilization, and blocking of the cells, the internalized TMab4 were stained with Alexa488-conjugated goat anti-human antibody, and subcellular endocytotic organelles were stained with anti-LAMP-1, followed by the appropriate TRITC-conjugated secondary antibody. The subsequent confocal microscopy was performed as described above.

2.6. Cytosolic calcein release assay

For tracing endosomal release of cytotransmabs or TAT using calcein [16,25], cells were incubated for 4 h with the indicated concentrations of antibodies or TAT in serum-free media at 37 °C using adalimumab as a non-internalizing negative control. Subsequently, 150 μM of calcein was added for 2 h at 37 °C. Cells were then washed three times with PBS and fixed. Calcein fluorescence images were obtained by confocal microscopy focusing on the center of cells at the same laser intensity for a set of samples. To analyze endosomal-released calcein, we selected the diffusive fluorescence signal area throughout the cytosolic region of cells, while excluding the punctuate fluorescence signal areas, and quantified the fluorescence intensity using ImageJ software. We analyzed more than 20 different cells for each sample and averaged the fluorescence intensity to get the calcein mean fluorescence intensity (MFI). The data were presented as the percentage of calcein MFI in the antibody/peptide-treated cells *versus* calcein MFI in the corresponding control cells.