Biochemical and Biophysical Research Communications 467 (2015) 771–777

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Quantitative assessment of cellular uptake and cytosolic access of antibody in living cells by an enhanced split GFP complementation assay

Ji-sun Kim [a], Dong-Ki Choi [a], Seong-wook Park [a], Seung-Min Shin [a], Jeomil Bae [a], Dong-Myung Kim [b], Tae Hyeon Yoo [a], Yong-Sung Kim [a, *]

[a] Department of Molecular Science and Technology, Ajou University, Suwon 443-749, South Korea
[b] Department of Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 305-764, South Korea

ARTICLE INFO

Article history:
Received 6 October 2015
Accepted 12 October 2015
Available online 19 October 2015

Keywords:
Cytosol-penetrating antibody
Cytosol localization
Endosomal escape
Split GFP
Streptavidin

ABSTRACT

Considering the number of cytosolic proteins associated with many diseases, development of cytosol-penetrating molecules from outside of living cells is highly in demand. To gain access to the cytosol after cellular uptake, cell-penetrating molecules should be released from intermediate endosomes prior to the lysosomal degradation. However, it is very challenging to distinguish the pool of cytosolic-released molecules from those trapped in the endocytic vesicles. Here we describe a method to directly demonstrate the cytosolic localization and quantification of cytosolic amount of a cytosol-penetrating IgG antibody, TMab4, based on enhanced split GFP complementation system. We generated TMab4 genetically fused with one GFP fragment and separately established HeLa cells expressing the other GFP fragment in the cytosol such that the complemented GFP fluorescence is observed only when extracellular-treated TMab4 reaches the cytosol after cellular internalization. The high affinity interactions between streptavidin-binding peptide 2 and streptavidin was employed as respective fusion partners of GFP fragments to enhance the sensitivity of GFP complementation. With this method, cytosolic concentration of TMab4 was estimated to be about 170 nM after extracellular treatment of HeLa cells with 1 μM TMab4 for 6 h. We also found that after cellular internalization into living cells, nearly 1.3–4.3% of the internalized TMab4 molecules escaped into the cytosol from the endocytic vesicles. Our enhanced split GFP complementation assay provides a useful tool to directly quantify cytosolic amount of cytosol-penetrating agents and allows cell-based high-throughput screening for cytosol-penetrating agents with increased endosomal-escaping activity.

1. Introduction

The needs of cytosolic delivery of antibodies and proteins are huge for targeting intracellular proteins in the cytosol [1,2]. However, antibodies and proteins cannot directly cross the plasma membrane due to their large size and hydrophilicity; but they can be internalized into cells primarily by receptor-mediated endocytosis [3]. The endocytosed molecules, including antibodies, usually follow the intracellular endosomal trafficking of early and late endosomes and lysosomes to be eventually degraded by the acidic pH and digestive enzymes [3,4]. To gain access to the cytosol, endocytosed molecules need to escape from the intermediate endosomal vesicles prior to the lysosomal degradation, also known as 'endosomal escape' [2,4].

For the characterization and development of cell-penetrating and cytosol-localizing molecules, herein referred to as 'cytosol-penetrating molecules' [5], it is practically very challenging to distinguish the pool of cytosolic released molecules from those trapped inside the endocytic vesicles [6]. The conventional fluorescence labeling and confocal microscopic analysis cannot absolutely differentiate the two pools [5,6]. Several approaches have been attempted to distinguish the cytosolic pool of internalized molecules from the endosomal pool, in the reporter cells which express enzymes to differentiate the cytosolic molecules from

Abbreviations: GFP, green fluorescence protein; IgG, immunoglobulin G; scFv, single-chain variable fragment; SA, streptavidin.

* Corresponding author. Dept. of Molecular Science and Technology, Ajou University, 206 Worldcup-ro, Yeongtong-gu, Suwon 443-749, South Korea.
E-mail address: kimys@ajou.ac.kr (Y.-S. Kim).

http://dx.doi.org/10.1016/j.bbrc.2015.10.066
0006-291X/

those in endosomes [4,6]. For example, a fluorescence probe conjugated to an internalizing protein is not fluorescent until hydrolyzed by β-galactosidase expressed only in the cytosol of cells [7]. A pH-sensitive fluorescent probe was developed to compare the relative cytosolic delivery efficiency of cell-penetrating peptides [8]. However, the chemical conjugation procedures might damage the intrinsic cell-penetrating properties of proteins and restrict its application in high-throughput screening [7]. Recently, the fusion of Cre recombinase to IgG was designed to monitor the cytosolic delivery of antibodies [6]. In this approach, cytosol-released Cre recombinase turned on the GFP gene expression in the reporter cell. However, continuous GFP expression amplifies the cytosolic delivery signals, indicating that it is not a quantitative method [9]. Biotinylation of Avi-tag fused cargo by cytosolically expressed BirA biotin ligase enabled easy comparison of cytosolic delivery efficiency of proteins [10]. Western blotting too has been used for this purpose, but it is not quantitative and is very time-consuming. Thus, a simple and direct assay is required for quantitative assessment of the amount of internalized molecules that are released into the cytosol from endocytic vesicles via endosomal escape.

We recently reported a cytosol-penetrating antibody of TMab4, also called cytotransmab [5]. Cytotransmab, TMab4 in its intact, full-length IgG form internalizes into living cells by clathrin-mediated endocytosis using cell-surface expressed heparan-sulfate proteoglycan (HSPG) as an internalizing receptor. Some fractions of internalized TMab4 escaped into the cytosol from early endosome without being further transported into other cellular compartments, such as lysosome, endoplasmic reticulum, Golgi apparatus, and nucleus [5]. However, the amount of cellular uptake and cytosolic delivery of antibody was not determined in detail. In this study, we developed a novel method to monitor the cytosolic delivery of TMab4 in living cells based on enhanced spit GFP complementation. We also estimated the amount of cellular uptake of TMab4 by quantitative Western blotting. Using the two quantitative assays, we assessed the endosomal escaping efficiency of cytosol-penetrating TMab4 antibody.

2. Material and methods

2.1. Cell lines and reagents

HeLa and HEK293T cells were purchased from American Type Culture Collection (ATCC), and maintained in DMEM supplemented with 10% FBS (HyClone), 100 units/mL penicillin, and 100 μg/mL streptomycin (Welgene) [5,11]. HEK293F cells were purchased from Invitrogen and maintained in FreeStyle 293 Expression Medium (Invitrogen). HRP-conjugated anti-mouse IgG (#7076) was obtained from Cell Signaling. HRP-conjugated anti-goat IgG (sc-2033) and anti-β-actin antibody (sc-69879) were obtained from Santa Cruz Biotechnology. Goat anti-human IgG (31125) antibody was purchased from Thermo scientific. Anti-FLAG antibody (F3165) was purchased from Sigma–Aldrich. All of the reagents were of analytical grade.

2.2. Construction of GFP11-SBP2-fused TMab4 scFv and IgG

For GFP complementation assay, engineered split GFP proteins composed of small GFP11 m3 fragment (strand 11, residues 215–230) and large GFP1-10 OPT fragment (strands 1–10, residues 1–214) were used [12]. Fusion of shortened streptavidin-binding peptide 2 (SBP2) [13] and streptavidin (SA) protein to the C-terminus of GFP11 and N-terminus of GFP1-10 fragments, respectively, were designed to enhance the efficiency of the GFP complementation. The SBP2 is a short 24 residue (GHVVEGLAGELEQLRARLEHHPQG) peptide, which binds to the naturally tetrameric SA molecule [13]. DNA fragment encoding the GFP11-$(G_4S)_3$ linker-SBP2 was prepared by DNA synthesis (Bioneer, Inc., Korea) with appropriate enzyme sites for subcloning. The GFP11-$(G_4S)_3$-SBP2 DNA fragment was subcloned in frame into TMab4 scFv encoding-pcDNA3.1 vector [5], generating pcDNA3.1-TMab4 scFv-GFP11-SBP2. This vector was designed to express TMab4 scFv-GFP11-SBP2 in the cell's cytosol. For the IgG expression of TMab4 fused to GFP11-SBP2 (TMab4-GFP11-SBP2), the GFP11-$(G_4S)_3$-SBP2 DNA fragment was subcloned in frame into the region encoding C-terminus of TMab4 heavy chain (HC) in pcDNA3.4-HC vector [5], generating pcDNA3.4-TMab4-GFP11-SBP2 HC vector. For light chain (LC) expression, we used previously constructed pcDNA3.4-LC vector [5].

2.3. Expression and purification of antibody

For IgG antibody expression of TMab4 and TMab4-GFP11-SBP2, the plasmids that encode the HC and LC were transiently co-transfected in pairs at an equivalent molar ratio into 0.2–1 L of HEK293F cell cultures in Freestyle 293F expression and media system (Invitrogen), as described before [5,11,14,15]. Antibodies were purified from the culture supernatants, as described [5,11,14,15] and their concentrations were determined using a Bicinchoninic Acid (BCA) Kit (Pierce, 23225).

2.4. Construction of SA-GFP1-10-expressing stable cell line by lentiviral transduction

DNA fragments encoding SA [13] and GFP1-10 OPT (residues 1–214) [12] were prepared by DNA synthesis (Bioneer, Inc., Korea) based on the sequence obtained from published papers. To construct SA-fused GFP1-10 (SA-GFP1-10) lentiviral expression vector, DNA fragment encoding SA-$(G_4S)_3$-GFP1-10 was subcloned in *SalI-Eco*RI enzyme site of pLJM lentiviral transfer vector, which has a FLAG-tag at the N-terminus. Lentiviral particles were produced in HEK293T cells as described before [16]. HeLa cells were infected with the viral particles and were selected with puromycin over 2 weeks [16]. More than 30 individual cells were screened for SA-GFP1-10 expression by Western blotting. Stable expressing SA-GFP1-10 cells were labeled as HeLa-SA-GFP1-10 cells.

2.5. Confocal microscopy

Confocal microscopy was performed as described before [5,11,14] for detection of antibodies inside cultured cells. Briefly, cells (4×10^4) were grown on 12-mm diameter coverslips and treated for 6 h with Alexa488-labeled TMab4, labeled with Alexa Fluor 488 antibody labeling kit (Molecular Probes, Invitrogen) [5]. After washing twice with PBS, the cells were washed with low-pH glycine buffer (200 mM glycine, 150 mM NaCl, pH 2.5) twice for 30 s, followed by 2 additional washes with PBS to remove non-internalized surface-bound antibodies [5]. The cells were fixed on the coverslips, stained with Hoechst 33342, and observed by confocal microscopy. Center-focused single z-section images were obtained on a Zeiss LSM710 system with ZEN software (Carl Zeiss). In experiment to test split GFP complementation in HeLa-SA-GFP1-10 cells by DNA transfection, pcDNA3.1-TMab4 scFv-GFP11-SBP2 plasmid was transfected with Lipofectamine™ 2000 (Invitrogen) into the HeLa-SA-GFP1-10 cells. After 24 h of growth, cells were subjected to confocal microscopy. In experiments of direct GFP fluorescence complementation by extracellular treatment with TMab4-GFP11-SBP2, HeLa-SA-GFP1-10 cells were incubated for 6 h at 37 °C with TMab4-GFP11-SBP2 and washed twice with low-pH glycine buffer. After fixation on coverslips and staining with Hoechst 33342, cells were subjected to confocal microscopy.

2.6. Western blotting

HeLa cells (2×10^5 cells) were incubated at 37 °C for 6 h with the indicated concentrations of TMab4 in 12-well culture plates, each with 500 μl of media. Cells were washed twice with the low-pH glycine buffer and PBS. After trypsinization, whole cell lysates were prepared in PBS [11,14]. Protein concentrations were measured using the BCA protein assay kit. Cell lysates were diluted with PBS to 12 μl volume containing ~10 μg of total proteins for loading on the SDS-PAGE gel followed by western blot analysis. To set up a standard western blotting plot with known amount of TMab4, known amounts (0–4 ng) of the purified TMab4 IgG was loaded on the same gel. Western blotting was performed with a goat anti-human IgG antibody followed by a HRP-conjugated anti-goat antibody. Proteins were visualized with PowerOpti-ECL Detection reagent (Animal Genetics lnc., Korea) and ImageQuant LAS 4000 mini (GE Health Care, Piscataway, NJ) [11,14]. Band densities were quantified using ImageJ software (National Institutes of Health, USA) [11,14]. The amount of TMab4 in cell culture media was also determined by western blotting with serially diluted culture media (about 1:12,500 dilution). All the western blotting exposures were performed under the same condition for direct comparisons.

2.7. Quantification of cytosolic TMab4-GFP11-SBP2 antibody

HeLa-SA-GFP1-10 cells (2×10^4 cells), grown in 96-well culture plates, were incubated at 37 °C for 6 h with 0.1, 0.5, 1 μM of TMab4-GFP11-SBP2 in 50 μl of media. After washing with PBS to remove autofluorescence of phenol red in media, 50 μl of PBS was added to each well. The complemented GFP signals from cells were measured with Synergy multi-mode microplate reader (BioTek, USA). As an intact GFP protein, GFP 11.3.3-L variant with improved folding efficiency was purified from *Escherichia. coli* cells as described before [17]. To set up GFP fluorescence standard plot with known amounts of intact GFP, various amounts (0–0.5 ng) of the purified, intact GFP were diluted 2-fold and added to the same 96-well plate in 50 μl of PBS. GFP fluorescence intensity was read at 528 nm after excitation at 485 nm [17]. The molar extinction coefficients (ε) of split GFP and intact GFP are 8.33×10^3 and $14.7 \times 10^3\ M^{-1}\ cm^{-1}$, respectively [17,18]. The quantum yield (Q) of split-GFP and intact GFP are 0.65 and 0.61, respectively. The brightness of GFP was calculated by the following formula:

$$\text{Brigtness of GFP} = \text{Quantum yied } (Q) \times \text{molar extinction coefficients } (\varepsilon) \quad (1)$$

We estimated the amount of split GFP taking into account of relative brightness of split GFP to intact GFP.

3. Results

3.1. Quantification of internalized amount of TMab4 cytotransmab

To visualize the cellular internalization and localization directly, Alexa488-labeled TMab4 cytotransmab was incubated with live HeLa cells under normal culture conditions for 6 h at 37 °C. Confocal microscopic analysis revealed that Alexa488-labeled TMab4 were seen throughout the cytoplasmic compartments with little accumulation in the nucleus (Fig. 1A), consistent with the previous results [5]. The internalized TMab4 were detected predominantly as intense punctate fluorescence and scarcely as diffused fluorescence, indicating that majority of them remain trapped within endocytic vesicles and only a minor portion reach the cytosol.

To determine the cellular uptake efficiency of TMab4, we measured the amount of internalized TMab4 in living HeLa cells by quantitative Western blotting after HeLa cells were incubated with various concentrations (0.1, 0.5, and 1 μM) of TMab4 at 37 °C for 6 h. The amount of internalized TMab4 (I) was quantified by fitting the antibody band intensities of cell lysates to the standard curve obtained with known antibody amounts (Fig. 1B and C), using the following formula:

$$\text{Internalized amount of TMab4 } (I) = \text{Amount of TMab4 in gel loaded cell lysate} \times \text{dilution factor } (D),$$
$$\text{where } D\ (\text{dilution factor}) = \frac{\text{The total volume of cell lysates}}{\text{The volume of gel loaded cell lysate}} \quad (2)$$

The estimated internalized amount of TMab4 was in sub-picomolar range (Table 1). We also estimated the amount of TMab4 in cell culture media at time = 0 (T) (immediately after antibody addition) and after 6 h of incubation (R) by quantitative western blot analysis of serially-diluted culture media and fitting the antibody band intensity to the standard curve (Fig. 1B and C). The cellular uptake efficiency (%) of TMab4 was determined:

$$\text{Cellular uptake efficiency } (\%) = \frac{I}{T} \times 100 \quad (3)$$

The estimated cellular uptake efficiency of TMab4 was 0.52, 0.29, and 0.22% following extracellular treatment of TMab4 at 0.1, 0.5, and 1 μM, respectively (Table 1). Lesser cellular uptake efficiency of TMab4 at the higher concentrations, typical for the receptor-mediated endocytosis was due to the receptor saturation and competition at the higher concentration [3]. We noticed that after 6 h of incubation, about 94–96% of TMab4 (R), compared with the initial amount (T) remained in cell growth media (Table 1). We previously noticed that TMab4 internalized within 30 min of incubation was degraded mostly likely via ubiquitin-proteasome-mediated proteolysis [5]. Thus, 4–6% loss of antibody seemed to be attributed to the degradation after cellular internalization during 6 h of treatment. Taking into account the cellular degradation of internalized antibody, we assumed the cellular uptake efficiency of TMab4 as approximately 3.7–5.7% of the initial treated amount of antibody (Table 1).

We also sought to estimate the intracellular concentrations of TMab4 by taking into account the volume of HeLa cells (~2000 μm^3) [15] and cell numbers in each well (2×10^5 cells) as:

$$\text{Intracellular concentration of TMab4} = I \div N \div V, \quad \text{where } N = \text{cell number},\ V = \text{cell volume} \quad (4)$$

The intracellular concentrations of TMab4 were estimated at ~0.50, 1.25, and 1.88 μM for the initial extracellular treatment with 0.1, 0.5, and 1 μM of TMab4, respectively (Table 1). Thus, though the cellular uptake efficiency seems low, the intracellular concentrations reached micromolar range.

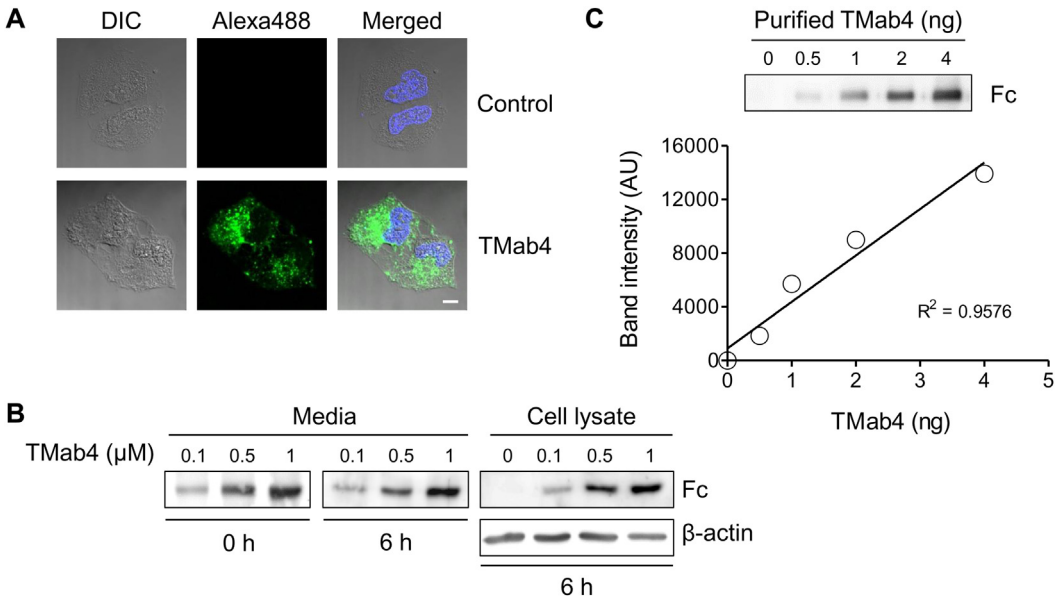

Fig. 1. Quantification of the cell-internalized TMab4 by western blotting. (A) Cellular internalization and localization of TMab4 in live HeLa cells treated with 1 μM of Alexa488-labeled TMab4 for 6 h at 37 °C and analyzed by confocal microscopy. Merged images show overlay of DIC (differential-interference-contrast), cell-penetrated TMab4 (green) and Hochest33342-stained nuclei (blue) in single confocal section at the center. Image magnification, 630×; scale bar, 10 μm. (B) Western blot analysis to quantify the amount of TMab4 in cell lysate or media in HeLa cells that were treated for 6 h at 37 °C with indicated concentrations of TMab4. Samples were diluted and ~10 μg of total protein was loaded on the SDS-PAGE gel followed by western blotting. β-Actin was used as a loading control. (C) Western blot analysis of known amounts (0–4 ng) of purified TMab4 to obtain the standard curve. The standard plot shows a linear regression of the amount of TMab4 *versus* the band intensities of the western blot measured by ImageJ software. The goodness of fit (R^2) is shown on the graph. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Design of an enhanced split GFP complementation assay in the cytosol of living cells

Quantitative western blot analysis with cell lysates allowed the estimation of total amount of internalized TMab4, but could not discriminate the antibody fraction in cytosol from that in endocytic vesicles. To estimate the amount of TMab4 that could reach the cytosol after internalization, we developed a one-step assay monitoring cytosolic localization of TMab4 with split GFP fluorescence complementation system [12]. For two split GFP fragments, we adopted the engineered GFP1-10 and GFP11 fragments [12] because they could be genetically fused into a protein with minimal effects on the solubility and conformation of the fused proteins [12]. Furthermore, we intended to increase the efficiency of GFP complementation in cytosolic environment of cells by fusing SBP2 peptide and SA protein to GFP11 and GFP1-10 fragments, respectively (Fig. 2A). Two molecules of SBP2 peptide bind tightly to one molecule of tetrameric SA with a dissociation constant (K_D) of ~1.5 nM [13]. Considering the orientation of SBP2 binding to SA in the complex crystal structure [13], we genetically linked SBP2 tag to C-terminus of GFP11 with $(G_4S)_3$ linker and fused SA to N-terminus of GFP1-10 fragment with $(G_4S)_3$ linker to induce optimal GFP fluorescence complementation (Fig. 2A).

To validate our design, we first established stable HeLa cells expressing SA-GFP1-10 in the cytosol with lentiviral particles that carry a SA-GFP1-10 fusion gene (Fig. 2B). We also constructed a cytosolic expression plasmid encoding TMab4-scFv-GFP11-SBP2, in which GFP11-SBP2 fragment fused to C-terminus of TMab4 scFv. Considering the cytosolic expression of the construct, we expressed TMab4 in the scFv format rather

Table 1
Summary of quantitative assessment of cellular uptake and cytosol penetrating efficiency of TMab4 antibody.

Parameters from quantitative western blot assay			
Treated concentration of TMab4 with HeLa cells (μM)	0.1	0.5	1
TMab4 in culture media at time = 0 h (T) (pmole)	40.3 ± 0.2	183 ± 2	348 ± 2
TMab4 in culture media (R) (pmole)[a]	(38 ± 2)	(174 ± 2)	(335 ± 5)
Internalized amount of TMab4 (I) (pmole)[a]	0.20 ± 0.01	0.50 ± 0.01	0.75 ± 0.02
Cellular uptake efficiency (%) = $I/T \times 100$[a]	0.52 ± 0.01	0.29 ± 0.01	0.22 ± 0.01
(Cellular uptake efficiency (%) = $100 - ((R + I)/T)$[a]	(5.7 ± 0.8)	(5.0 ± 2.1)	(3.7 ± 0.9)
Intracellular concentration of TMab4 (μM)[a]	0.50 ± 0.01	1.25 ± 0.01	1.88 ± 0.01
Parameters from split GFP complementation assay			
Treated concentration of TMab4-GFP11-SBP2 with HeLa-SA-GFP1-10 cells (μM)	0.1	0.5	1
Cytosolic amount of TMab4 (fmole)[b]	0.24 ± 0.17	1.23 ± 0.20	3.21 ± 0.44
Endosome-escaping efficiency (%)[b]	1.3 ± 0.8	2.5 ± 0.5	4.3 ± 0.6
Cytosolic concentration of TMab4 (nM)[b]	13 ± 2	65 ± 2	171 ± 4

[a,b]Western blot and split GFP fluorescence complementation assays were measured after 6 h of incubation of antibodies with cells under the same ratio of molar amount of antibody to the total number of cells. The values represent mean ± SD of at least three independent experiments.

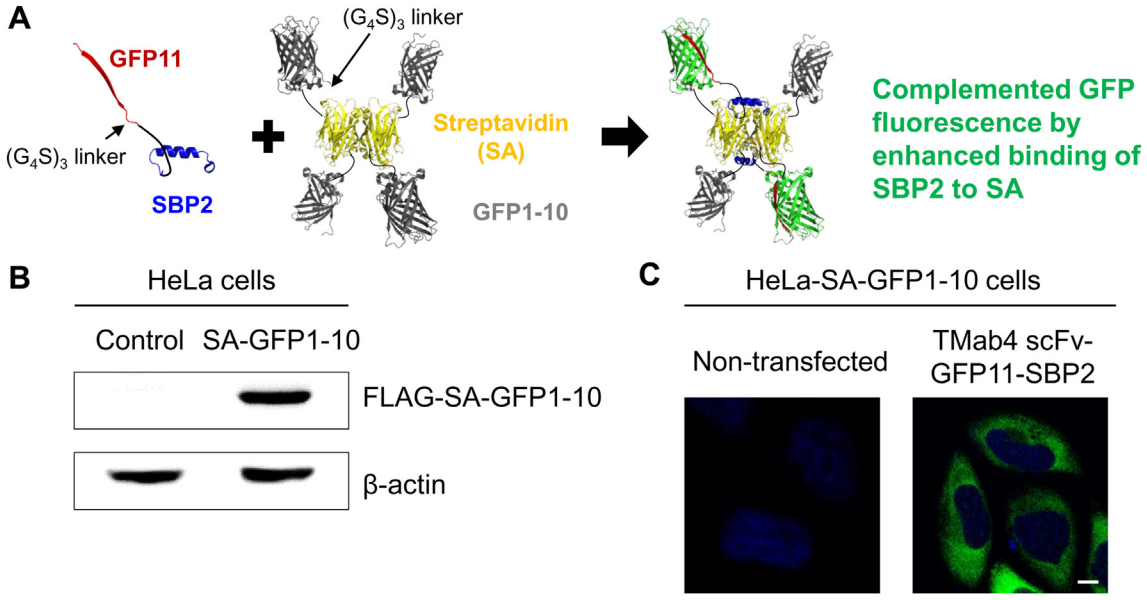

Fig. 2. Design and validation of enhanced split GFP complementation assay in the cytosol of living cells. (A) Schematic diagram of the enhanced split GFP complementation assay. SBP2 peptide and SA protein was fused to the C-terminus of GFP11 and N-terminus of GFP1-10 fragments using $(G_4S)_3$ linker, respectively, for productive GFP fluorescence complementation by SBP2 and SA interaction. (B) Western blotting of cell lysates of wild type (control) and established HeLa-SA-GFP1-10 cells. β-Actin was used as a loading control. (C) GFP complementation was verified by transiently transfecting the plasmid encoding TMab4-scFv-GFP11-SBP2 into HeLa-SA-GFP1-10 cells. Control shows non-transfected HeLa-SA-GFP1-10 cells. Image magnification, 400×; scale bar, 10 μm.

than the IgG format. When the TMab4-scFv-GFP11-SBP2 plasmid transfected into HeLa-SA-GFP1-10 cells, the complemented GFP fluorescence was detected strongly only from the cytosolic regions of the cells (Fig. 2C). Cells untransfected with HeLa-SA-GFP1-10 gave no GFP fluorescence signals. These data demonstrate that cytosolically expressed TMab4-scFv-GFP11-SBP2 complements properly with SA-GFP1-10 fragment. The complemented GFP fluorescence was detected as discontinuous, tiny punctate pattern rather than completely diffusive pattern. We reasoned that the GFP complementation in the oligomeric complex due to the binding of two molecules of GFP11-SBP2 to one molecule of tetrameric SA-GFP1-10 (Fig. 2A) resulted in the discontinuous GFP fluorescence, as was observed before [19].

To quantify the amount of TMab4-GFP11-SBP2 in the cytosol, we treated HeLa-SA-GFP1-10 cells with 0.1, 0.5, 1 μM of TMab4-GFP11-SBP2 for 6 h at 37 °C in 96-well plates and directly measured GFP fluorescence with a spectrofluorometer. In this fluorescence assay, the ratio of molar amount of antibody to the total number of cells was kept same with that of the western blotting for uniformity in analysis of the results. The total quantity of cytosolic TMab4-GFP11-SBP2 was calculated by fitting the intensity of GFP fluorescence to a standard curve obtained from serially diluted intact GFP (Fig. 3C). Since the molar amount of assembled split-GFP is equal to that of TMab4-GFP11-SBP2 (Fig. 3A), we assumed the cytosolic of TMab4 is equivalent to amount of split GFP obtained after normalization with the relative brightness factor of split GFP to that of intact GFP [17,18]. Thus, the cytosolic amount of TMab4 was estimated as follows:

$$\textit{Cytosolic amount of TMab4}\ (C) = \textit{Cytosolic amount of split GFP}\ \left(C^{'}\right) \times \frac{\textit{Brightness of intact GFP}}{\textit{Brightness of split GFP}} \tag{5}$$

3.3. Quantification of cytosolic TMab4 cytotransmab after the internalization

With the validated split GFP complementation assay, we first sought to demonstrate the cytosolic access of TMab4 in full-length IgG format. We purified TMab4-GFP11-SBP2 antibody, in which GFP11-SBP2 was fused to C-terminus of heavy chain of TMab4. As shown in Fig. 3A, we could detect complemented GFP fluorescence only when extracellular TMab4-GFP11-SBP2 reaches the cytosol of HeLa-SA-GFP1-10 cells and assembles with SA-GFP1-10 there. When the purified TMab4-GFP11-SBP2 was incubated at 37 °C for 6 h with HeLa-SA-GFP1-10 cells under normal cell culture conditions, complemented GFP fluorescence was observed in the cytosol in proportion to the concentrations of TMab4-GFP11-SBP2 (Fig. 3B), thus clearly indicating that TMab4 has the ability to reach the cytosol.

The cytosolic amount of TMab4 was in femtomolar range with increase in proportion to the initial extracellular concentrations (Table 1). We estimated endosomal escaping efficiency (%) of TMab4 by dividing the cytosolic amount of TMab4 (C) with the internalized amount of TMab4 (I) obtained from the equation (2) following quantitative Western blotting:

$$\textit{Endosomal escaping efficiency}\ (\%) = \frac{\textit{Cytosolic amount of TMab4}\ (C)}{\textit{Internalized amount of TMab4}\ (I)} \times 100 \tag{6}$$

The endosomal escaping efficiency of TMab4 was about 1.3, 2.5, and 4.3% for the initial extracellular TMab4 concentrations of 0.1, 0.5, and 1 μM, respectively (Table 1). A better endosomal escape efficiency of TMab4 at higher concentrations indicates that the

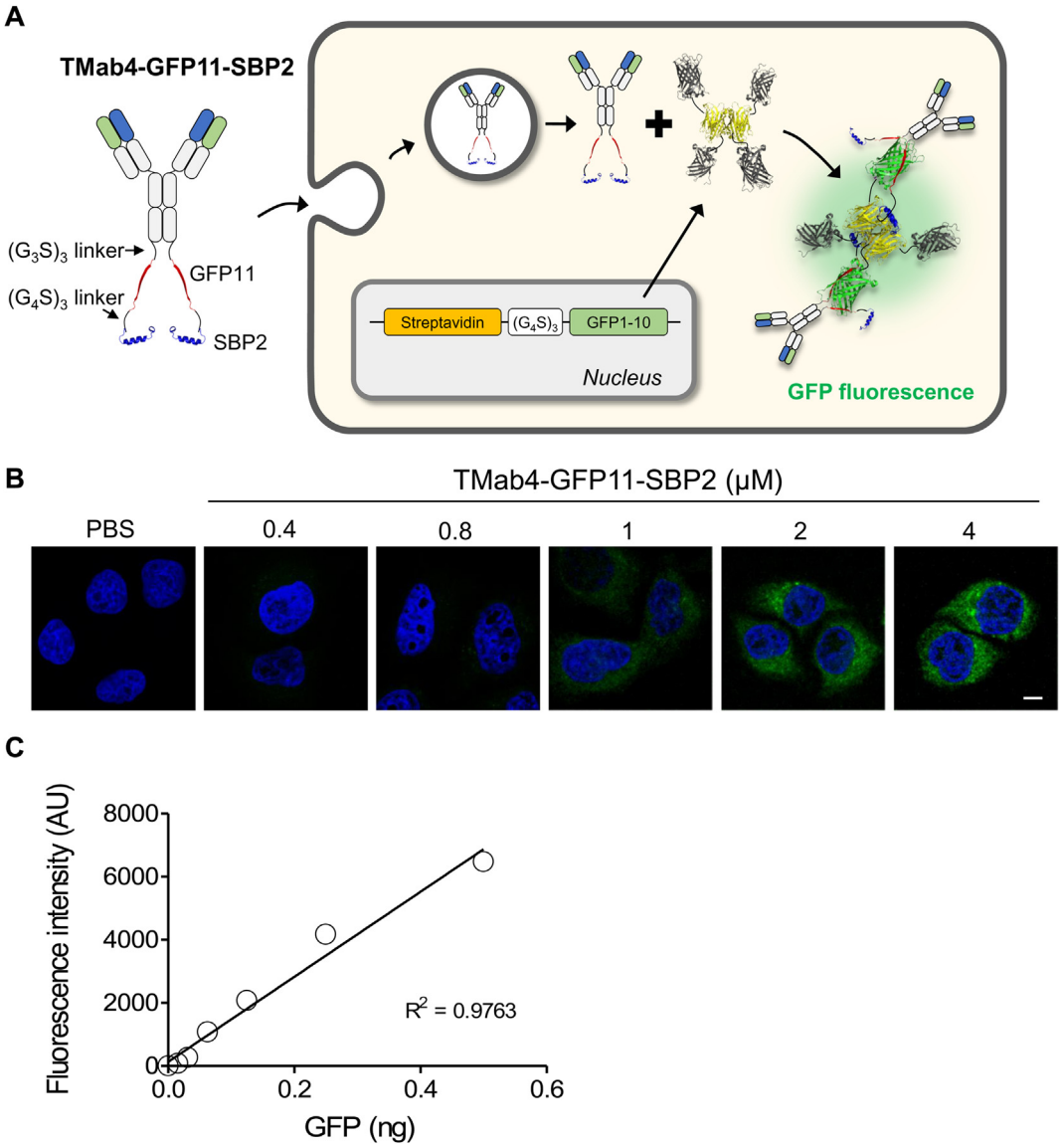

Fig. 3. Quantification of cytosolic access of TMab4 by the enhanced split GFP complementation assay. (A) Schematic diagram showing GFP fluorescence complementation, which occurs only when extracellularly treated TMab4-GFP11-SBP2 internalizes into living HeLa-SA-GFP1-10 cells, escapes from the endocytic vesicles into the cytosol, and assembles with the SA-GFP1-10 proteins. (B) Confocal microscopic analysis of complemented GFP signals in HeLa-SA-GFP1-10 cells, which were treated at 37 °C for 6 h with the indicated concentrations of TMab4-GFP11-SBP2. Image magnification, 630×; scale bar, 10 μm. (C) Standard plot for GFP fluorescence showing linear regression of the amount of intact GFP (0–0.5 ng) *versus* the fluorescence intensities. The goodness of fit (R^2) is shown on the graph.

endosomal escape of TMab4 from endocytic vesicles into cytosol is most likely mediated by the oligomerization mechanism, which is concentration-dependent like the mechanism of pore formation [20].

We further estimated the molar concentration of the TMab4 in cytosol by taking into account the cytosolic volume of HeLa cells (~940 μm^3) [21] and cell numbers in each well (2×10^4 cells) as follows:

$$\begin{aligned} &\textit{Cytosolic concentration of TMab4} = C \div N \div V, \\ &\textit{Where } N = \textit{cell number},\ V = \textit{cell volume} \end{aligned} \tag{7}$$

As shown in Table 1, the cytosolic concentration of TMab4 was about 13, 65, and 171 nM for the initial extracellular TMab4 treatments at 0.1, 0.5, and 1 μM, respectively, displaying a dose-dependent increment of cytosolic concentration.

4. Discussion

In studies with cell-penetrating molecules, it is very challenging to demonstrate directly their cytosolic access after cellular internalization and to quantify their cytosolic amount by distinguishing from the fraction trapped in the endocytic vesicles. In our study, we developed a novel method to directly demonstrate the cytosolic localization and quantification of the cytosolic amount of TMab4 cytotransmab by designing spit GFP complementation, which occurs only when the extracellularly treated TMab4 reaches the cytosol of living cells. To our knowledge, this is the first report of quantitative assessment of cytosolic access and endosomal escape efficiency of cell-penetrating antibody in living cells.

Genetic fusion of the split GFP fragments simplified the recombinant antibody preparation, skipping costly and complicated chemical ligation steps. Given that only a small fraction of internalized TMab4 escapes into the cytosol, the high affinity

interactions ($K_D \approx 1.5$ nM) between SBP2 and SA was exploited for the respective fusions to the GFP fragments to enhance the sensitivity of GFP complementation in the crowded cytosolic environment of living cells. Furthermore, the spontaneous GFP fluorescence via two-fragment complementation does not require any post sampling steps such as cell lysis and cell fixation. Thus, our enhanced split GFP complementation assay composed of living cells expressing one GFP fragment in the cytosol and cell-penetrating molecules fused with the other GFP fragment will permit cell-based high-throughput screening of cytosol-penetrating antibodies or peptides with improved endosomal-escaping activity. Further, time-course monitoring of GFP fluorescence in the living cells could provide information on spatiotemporal intracellular trafficking of cytosol-penetrating agents.

Technology of enabling extracellular antibodies to reach the cytosol of living cells is emerging to target numerous disease-associated cytosolic proteins [1,6]. Cytotransmab TMab4 is a new class of antibody that penetrates into the cytosol of living cells [5]. After internalization into living cells, only a small fraction (1.3–4.3%) of TMab4 entrapped in the endosomal vesicles was released into the cytosol (Table 1). After 6 h of incubation, however, the actual cytosolic concentration of TMab4 was estimated to be about 13, 65, and 171 nM after initial extracellular treatment with 0.1, 0.5, and 1 μM of TMab4, respectively (Table 1). This indicates that, although a small portion of internalized TMab4 reached the cytosol, the cytosolic concentration of TMab4 would be competent to target cytosolic proteins if we engineer TMab4 to have nanomolar range affinity with the targeted antigen [5].

Taken together, our enhanced split GFP complementation assay provides a useful tool to directly quantify cytosolic amount of cytosol-penetrating molecules and their cell-based high-throughput screening for the improved endosomal-escaping activity.

Acknowledgments

This study was supported by grants from the Pioneer Research Center Program (2014M3C1A3051470), the Mid-career Researcher Program (2013R1A2A2A01005817), and the Converging Research Center Program (2009–0093653) of the National Research Foundation, funded by the Korean government.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.10.066.

References

[1] A.A. Ivanov, F.R. Khuri, H. Fu, Targeting protein-protein interactions as an anticancer strategy, Trends Pharmacol. Sci. 34 (2013) 393–400.
[2] S. Guillard, R.R. Minter, R.H. Jackson, Engineering therapeutic proteins for cell entry: the natural approach, Trends Biotechnol. 33 (2015) 163–171.
[3] M. Ritchie, L. Tchistiakova, N. Scott, Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates, mAbs 5 (2013) 13–21.
[4] A. Fu, R. Tang, J. Hardie, et al., Promises and pitfalls of intracellular delivery of proteins, Bioconjug. Chem. 25 (2014) 1602–1608.
[5] D.K. Choi, J. Bae, S.M. Shin, et al., A general strategy for generating intact, full-length IgG antibodies that penetrate into the cytosol of living cells,, mAbs 6 (2014) 1402–1414.
[6] A.L. Marschall, C. Zhang, A. Frenzel, et al., Delivery of antibodies to the cytosol: debunking the myths, mAbs 6 (2014) 943–956.
[7] T.Y. Chao, R.T. Raines, Fluorogenic label to quantify the cytosolic delivery of macromolecules, Mol. Biosyst. 9 (2013) 339–342.
[8] Z. Qian, P.G. Dougherty, D. Pei, Monitoring the cytosolic entry of cell-penetrating peptides using a pH-sensitive fluorophore, Chem. Commun. Camb. 51 (2015) 2162–2165.
[9] J.J. Cronican, K.T. Beier, T.N. Davis, et al., A class of human proteins that deliver functional proteins into mammalian cells in vitro and in vivo,, Chem. Biol. 18 (2011) 833–838.
[10] W.P. Verdurmen, M. Luginbuhl, A. Honegger, et al., Efficient cell-specific uptake of binding proteins into the cytoplasm through engineered modular transport systems, J. Control Release 200 (2015) 13–22.
[11] H.J. Choi, Y.J. Kim, S. Lee, et al., A heterodimeric Fc-based bispecific antibody simultaneously targeting VEGFR-2 and Met exhibits potent antitumor activity,, Mol. Cancer Ther. 12 (2013) 2748–2759.
[12] S. Cabantous, T.C. Terwilliger, G.S. Waldo, Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein, Nat. Biotechnol. 23 (2005) 102–107.
[13] I.H. Barrette-Ng, S.C. Wu, W.M. Tjia, et al., The structure of the SBP-Tag-streptavidin complex reveals a novel helical scaffold bridging binding pockets on separate subunits, Acta Crystallogr. Sect. D. Biol. Crystallogr. 69 (2013) 879–887.
[14] Y.J. Kim, J. Bae, T.H. Shin, et al., Immunoglobulin Fc-fused, neuropilin-1-specific peptide shows efficient tumor tissue penetration and inhibits tumor growth via anti-angiogenesis, J. Control Release 216 (2015) 56–68.
[15] D.S. Baek, Y.S. Kim, Humanization of a phosphothreonine peptide-specific chicken antibody by combinatorial library optimization of the phosphoepitope-binding motif, Biochem. Biophys. Res. Commun. 463 (2015) 414–420.
[16] X. Wang, M. McManus, Lentivirus production, J. Vis. Exp. 32 (2009) e1499, http://dx.doi.org/10.3791/1499.
[17] T.H. Yoo, A.J. Link, D.A. Tirrell, Evolution of a fluorinated green fluorescent protein, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 13887–13890.
[18] J.D. Pedelacq, S. Cabantous, T. Tran, et al., Engineering and characterization of a superfolder green fluorescent protein, Nat. Biotechnol. 24 (2006) 79–88.
[19] C. Oker-Blom, A. Orellana, K. Keinanen, Highly efficient production of GFP and its derivatives in insect cells for visual in vitro applications, FEBS Lett. 389 (1996) 238–243.
[20] A.K. Varkouhi, M. Scholte, G. Storm, et al., Endosomal escape pathways for delivery of biologicals, J. Control Release 151 (2011) 220–228.
[21] A. Fujioka, K. Terai, R.E. Itoh, et al., Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes, J. Biol. Chem. 281 (2006) 8917–8926.