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An anti-peptide monoclonal antibody recognizing the tobacco etch virus protease-cleavage sequence and its application to a tandem tagging system

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ABSTRACT

Peptide-based affinity tags are commonly used in recombinant production/purification of proteins, and are often preceded or followed by a protease recognition sequence to allow tag removal. We describe a rat monoclonal antibody 2H5 recognizing an undecapeptide tag called "eTev", which contains a recognition sequence for Tobacco Etch Virus (TEV) protease. In the crystal structure of 2H5-eTev complex, the long eTev peptide assumes compact α-helical conformation in the binding groove, exposing both ends to the solution. This architecture allowed us to connect eTev with another peptide tag called PA tag via linker sequence, ensuring the simultaneous access of two anti-tag antibodies. When this tandem double tag was attached at one end of various proteins, it enabled highly sensitive and protein-independent detection by sandwich ELISA. Utilizing this system during a rapid cell line screening, we succeeded in isolating stable cell clones expressing high level of mouse Wise protein.

Recombinant production and purification of proteins for their functional and structural analyses has become a routine practice in the biomedical research field. In most cases, the protein of interest is genetically fused with artificial sequences that code for "affinity tag" at either end, enabling detection, labeling, and purification of the target protein. Peptide-based epitope tag is particularly convenient because of the small size of the tag, and many excellent epitope tag systems comprising tag peptide and anti-peptide antibody are available [reviewed in Ref. [1]]. In certain applications it is desired to attach two or more epitope tags to one protein so that each affinity purification/detection procedure can be done in a sequential manner [2]. "Double tagging" also enables a sandwich ELISA, where one antibody is used to capture a protein to a solid support and the other antibody is used for detection [3]. This is particularly useful when there is no good antibody available for the target protein, but the two tags must be well separated in space to ensure the simultaneous binding of the two antibodies.

Even though the epitope tags are short and generally do not interfere with the function of the target protein, it is often necessary to remove the artificially added tag sequence after the purification, particularly when the protein is to be used in a crystallographic projects [4]. Inclusion of a protease-recognition sequence in between the protein and the tag portion is the strategy routinely employed in such cases [5]. When multiple tagging is desired, however, putting the protease site in each tag portion will require more complex construct design and may result in unexpected effect on the function of the protein due to the longer artificial sequence to be attached. One potential way to get around this problem is to use the protease-recognition sequence itself as a tag portion. In fact, the FLAG tag (DYKDDDDK) originally developed by Hopp and colleagues contains a recognition sequence for a protease enterokinase and potentially can be used is such application [6]. However, the commercially available enterokinase is relatively expensive and does not have highly strict recognition specificity toward the tag sequence, making the FLAG tag rarely used for a cleavage purpose despite its wide usage in the general tagging purposes. In contrast to enterokinase, tobacco etch virus (TEV) protease can be easily produced recombinantly in *E. coli*, has very high recognition specificity toward 7-residue sequence (ENLYFQG), and the cleavage reaction can proceed at 4° [7,8]. In this paper, we succeeded in establishing

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an anti-peptide monoclonal antibody whose epitope includes the TEV protease recognition sequence. This tag, dubbed eTev tag, was combined with another peptide (PA tag) [9] to make a single stretch of polypeptide carrying two closely positioned epitopes in tandem. Crystal structure of the tag-antibody complex was used to optimize the linker length between the PA-eTev or eTev-PA tandem that allows simultaneous binding of two antibodies, resulting in a development of a single cleavable tag compatible with semi-quantitative sandwich ELISA without the need for any antibodies specific for the target protein.

1. Materials and methods

1.1. Production of a monoclonal antibody

An 11-amino-acid peptide (RENLYFQGKDC) having the TEV protease recognition sequence (underlined) and charged amino acids at both ends was synthesized using Fmoc chemistry, followed by a conjugation to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) through the cysteine located at the C-terminus. Immunization was performed by intracutaneous administration of the peptide-KLH complex together with an adjuvant (100 μL per foot, 200 μL/animal in total) into the soles of rear feet of a Sprague Dawley rat (female, 8 weeks old). Two weeks after the immunization, the antibody titer was measured by ELISA. The rat iliac lymphocytes were fused with mouse myeloma cells (SP2/0 cell line) by the polyethylene glycol method. Hybridomas were selected in complete RPMI1640-10% fetal calf serum (FCS) with HAT supplement and subjected to limiting dilution culture in 96-well plates, followed by culture in HT medium. Supernatants were initially screened for antibodies with ELISA using peptide-BSA conjugate as antigen, and later using a fusion protein described below. Resultant hybridoma clone (2H5) produces IgG2a (κ), and the IgG was purified from hybridoma culture supernatants using recombinant Protein G-Sepharose (GE Healthcare). N-terminal as well as several internal amino acid sequences were obtained by Edman sequencing of the purified antibody using a Shimadzu Protein Sequencer PPSQ-51A/53A (Shimadzu Corp.), and the heavy and light chain genes were cloned as described previously [10].

1.2. Fusion protein

To test the reactivity of 2H5 toward eTev sequence, fusion proteins containing the eTev peptide were prepared. To this end, the antigen sequence or its variants were fused to the N-terminus of a fragment of human fibronectin comprising of the 9th to 10th type III repeats [11]. The peptide segments were prepared by extension PCR and the entire coding region was cloned into the NdeI-BamHI site of the pET-16b vector (Novagen), resulting in the N-terminally 10xHis-tagged eTev-Fn proteins. To validate the “tandem double tag” application, a 161-residue model protein T4 lysozyme (T4L) was fused either N-terminally with a 29-residue peptide with the following sequence; GVAMPGAEDDVVGGGGNSRENLYFQGKDG (dubbed nPAW tag for “n-terminal PA-containing double(W) tag”) or C-terminally with a 30-residue peptide with the following sequence; RENLYFQGKDGGGGGSHMGVAMPGAEDDVV (dubbed cPAW tag for “c-terminal PA-containing W tag”), and cloned into the NdeI-BamHI site of the pET16b vector as above. All these recombinant proteins were expressed in *E. coli* using the BL21(DE3) strain as a host, and purified from the soluble fraction of the bacterial lysate by Ni-NTA chromatography. Two more proteins, mouse neuropilin-1 ectodomain (Nrp1ec) and human serum albumin (HSA), were also appended with cPAW tag (Fig. 3A) and produced in mammalian expression system in a similar way described previously [9].

1.3. ELISA

For the direct ELISA assay, microtiter plates (NUNC Maxisorp) were coated overnight at 4 °C with eTev-Fn fusion proteins (10 μg/ml) or eTev peptide (30 μg/ml) and blocked with 5% skim milk in Tris-buffered saline (TBS). Hybridoma supernatants or purified IgG serially diluted in TBS containing 1% BSA were then added and incubated for 1 h at room temperature, and wells were washed 3 times with 200 μL TBS. To detect bound antibody, horse radish peroxidase (HRP)-conjugated goat anti-rat secondary antibody (Southern Biotech) diluted to 1:1000 in TBS was added and incubated for 30 min at room temperature, and wells were washed 4 times with TBS. Color was developed by the addition of 100 μL of ABTS substrate solution (KPL) and absorbance at 405 nm was recorded using a multiplate reader (Emax, Molecular Devices).

For the sandwich ELISA, microtiter plates were coated overnight at 4 °C with anti-PA tag antibody NZ-1 (Wako Pure Chemical Co., 20 μg/ml), followed by blocking with 5% skim milk in TBS. The test samples containing the proteins tagged with either nPAW or cPAW peptides were then added to the wells and incubated for > 3 h at 4 °C to allow the completion of the capture reaction. After washing the wells 3 times with 200 μL TBS, biotinylated 2H5 IgG (10 μg/ml in TBS containing 0.1% BSA) was added and incubated for 1 h at room temperature. To detect bound 2H5, HRP-conjugated streptavidin (VECTOR) diluted to 1:1000 in TBS containing 0.1% BSA was added and incubated for 30 min at room temperature, followed by the TBS wash and color development as described above.

1.4. TEV cleavage assay

Various mutant eTev-Fn (400 μg/ml) was incubated with TEV protease at an enzyme/substrate ratio of 1:10 (wt/wt) in TBS for 16 h at 20 °C. The reaction was terminated by the addition of SDS-PAGE sample buffer and analyzed by SDS-PAGE. TEV cleavage was judged by the conversion of intact eTev-Fn band (22 kDa) to a fast-migrating 19 kDa band on a 12% polyacrylamide gel.

1.5. Binding analysis using surface plasmon resonance (SPR)

Kinetic binding analysis was performed using BIACORE 2000 (GE Healthcare) as described previously [9]. First, purified 2H5 IgG was immobilized on a CM5 sensor chip using amino coupling chemistry at about 1000 RU according to the method provided by the manufacturer. The eTev-Fn proteins were diluted in a running buffer (TBS containing 0.005% Tween 20, TBST) and injected at a flow rate of 20 μl/min. Kinetic binding parameters were derived from the sensorgram curves by global fitting to a 1:1 Langmuir model using the BIAevaluation software (GE Healthcare).

1.6. Preparation and crystallization of 2H5 Fab fragment

2H5 IgG was purified from hybridoma culture supernatants using recombinant Protein G-Sepharose (GE Healthcare). Purified IgG was dialyzed against PE buffer (20 mM Na/K phosphate, 10 mM EDTA, pH 7.0) and incubated with Immobilized Papain (Thermo Scientific) (0.1 ml per mg IgG) in the presence of 25 mM Cys-HCl (pH 7.0) for ~4 h at 37 °C under gentle agitation. After the removal of the Immobilized Papain, the reaction mixture was dialyzed against TBS, and slowly added to eTev peptide-conjugated Sepharose. Bound Fab was eluted with 0.1 M Gly-HCl, pH 2.3, dialyzed against TBS, and further purified by gel filtration chromatography on a Superdex 200 16/60 column equilibrated with TBS. The concentrated Fab solution (10.5 mg/ml) was mixed with 1.0 mM eTev peptide, and the crystallization screening of the Fab-peptide complex was carried out using the hanging drop vapor-diffusion method, resulting in many hit conditions. Upon the optimization, the diffraction quality crystals were grown under the condition of 0.1 M Tris-HCl pH 7.0, 20% polyethylene glycol monomethyl ether 2,000, 0.5 M NaCl.

Table 1
X-ray data diffraction statistics and Refinement statistics.

Diffraction data statistics	
Space group	$P3_221$
Unit cell parameters	$a = b = 217.8$ Å, $c = 52.2$ Å
No. Molecule/ASU	2
Resolution [Å]	50.0–2.05 (2.12–2.05)
No. Unique Reflections	88376 (8730)
Multiplicity	5.3 (5.1)
Completeness [%]	99.5 (98.9)
R_{sym} [a]	0.148 (0.491)
Mean $I/\sigma(I)$	21.0 (5.2)
Wilson B factor	21.9
Refinement statistics	
Resolution Range [Å]	45.7–2.05 (2.10–2.05)
No. of nonhydrogen atoms	7201
No. of water molecules	591
R_{work}/R_{free} [b]	0.189/0.221 (0.241/0.273)
RMSD bond length [Å]	0.012
RMSD bond angle [°]	1.511
Ramachandran plot	
In most favored regions [%]	97.7
In disallowed regions [%]	0.00

Values in parentheses refer to the highest resolution shell.
[a] $R_{sym} = \Sigma|I_{hkl} - \langle I_{hkl}\rangle|/\Sigma I_{hkl}$.
[b] $R_{work} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, R_{free} was calculated from the test set (5% reflections of the total data).

1.7. X-ray data collection and structural determination

The diffraction data sets were collected at BL32XU beamline, SPring-8 (Hyogo, Japan) with the helical data collection mode [12]. The diffraction images were processed with HKL2000 [13], and the initial phases were calculated by molecular replacement method with PHASER [14] using a Fab structure of IgG from rat (PDID: 1BFO) as a search model. The resultant model was refined using REFMAC5 [15] with manual editing on COOT [16]. The model validation was carried out with MOLPROBITY [17]. Diffraction data statistics and final refinement statistics are shown in Table 1. All structural figures were prepared with the program PyMOL (Schrödinger, LLC). Atomic coordinates and structural factors have been deposited in the Protein Data Bank under an accession number 5AUM.

1.8. Establishment of stable cell line expressing mouse Wise

A plasmid DNA encoding nPAW-tagged mouse Wnt modulator in surface ectoderm (Wise) in the pcDNA3.1 backbone was co-transfected with a plasmid containing puromycin resistance gene into Expi293F cells (Thermo Fisher Scientific) and plated into multiple 96-well plates. Cells were cultured for 5 weeks in a medium containing 1 mg/mL geneticin (G418) and 10 μg/mL puromycin to select for stable integrants. Culture supernatants from wells containing single colony were assessed for the presence of nPAW-Wise using the sandwich ELISA described above, and positive clones were expanded and further evaluated for the expression level after several passages. Several cell clones stably maintaining the high expression level over a long culture period were obtained.

2. Results and discussion

2.1. Isolation of anti-eTev tag antibody 2H5 and its epitope mapping

The TEV protease is known to recognize a stretch of 7 amino acids (ENLYFQG) and cleaves between Gln and Gly [8]. The unusually long recognition sequence makes this endopeptidase highly unique and useful because the chance of unwanted cleavage within the target protein at positions other than the engineered site is very low. For the production of monoclonal antibodies recognizing this sequence,

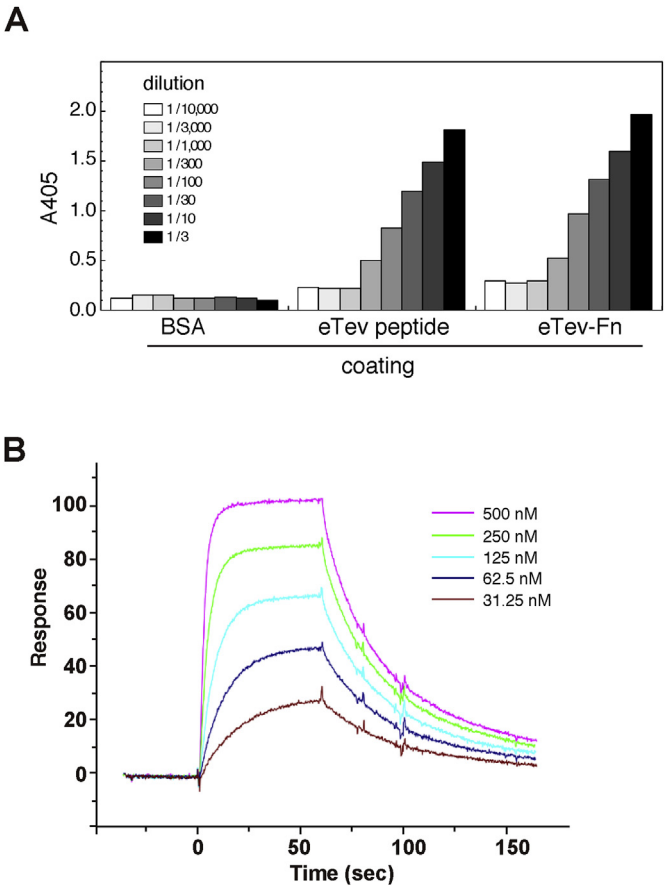

Fig. 1. Reactivity of 2H5 antibody toward eTev sequence. (A) Direct ELISA. Serially diluted 2H5 hybridoma culture supernatants were incubated with wells coated with indicated proteins. Bindings were evaluated by measuring the absorbance at 405 nm after addition of chromogenic substrate to the wells. (B) Surface plasmon resonance analysis. Serially diluted eTev-Fn proteins (31.25, 62.5, 125, 250, and 500 nM) were injected over the CM5 sensor chip immobilized with 2H5 for 60 s, followed by dissociation in TBST for 120 s at a flow rate of 20 μl/min.

however, we suspected that seven residues may not be long enough to elicit specific and strong immune reaction when immunized in rats. So we extended the peptide at both sides with charged residues, and used a decapeptide (RENLYFQGKD, dubbed eTev for extended TEV recognition sequence) as an immunogen after conjugation with KLH through an extra cysteine placed at the C-terminus. Using a standard hybridoma technology, a rat-mouse hybridoma clone 2H5 secreting rat IgG_{2a}/kappa antibody was isolated. The culture supernatants from 2H5 showed strong reactivity in ELISA assays with the eTev peptide used in the immunization as well as the eTev-Fn fusion protein directly coated onto the microtiter wells, with virtually no binding toward control BSA-coated surface (Fig. 1A). We also analyzed the kinetic binding behavior of purified 2H5 antibody toward eTev-Fn by using surface plasmon resonance, and determined the kinetic parameters as $k_{on} = 5.02 \times 10^5$ $M^{-1}s^{-1}$ and $k_{off} = 0.0359\,s^{-1}$, resulting in the K_D value of 71.4 nM (Fig. 1B). Due to the rather fast dissociation rate, eTev-2H5 system may not be ideal for the protein purification purpose. However, the affinity is high enough to be applied in an ELISA-based detection/quantification procedures, as will be discussed in the later sections. In order to define the sequence specificity of the recognition, we performed alanine-scanning experiment using the eTev-Fn mutants. Fig. 2A shows the concentration dependent binding of purified 2H5 IgG toward various Ala mutant eTev-Fn proteins. Ala substitutions at R1, L4, Y5, G8, K9, and D10 had strong negative effect on the 2H5-reactivity resulting in a partial to complete elimination of the binding, indicating the critical involvement of these residues in the recognition. Ala substitutions at

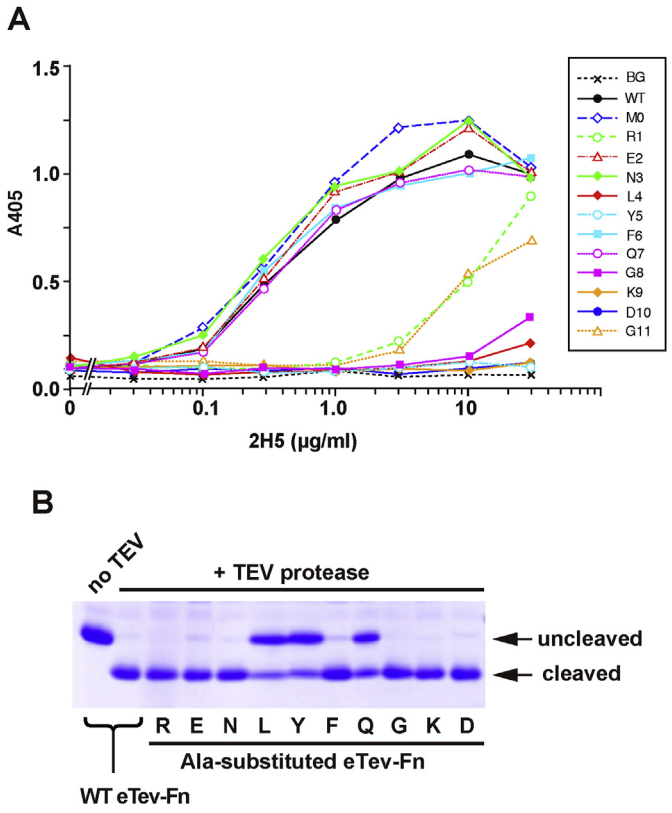

Fig. 2. Alanine scanning mutagenesis. (A) Sequence specificity of the recognition by 2H5. Mutant eTev-Fn proteins containing Ala-substitution at the indicated amino acid position was coated onto the microtiter wells, and concentration-dependent binding of 2H5 IgG was evaluated by direct ELISA as in Fig.1A. BSA-coated wells were used as background (BG) control. (B) Sequence specificity of recognition by TEV protease. The Ala-substituted mutant eTev-Fn proteins were incubated with TEV protease at enzyme:substrate ratio of 1:10 for 16 h. Cleavage reaction was monitored by SDS-PAGE as the conversion of the eTev-Fn band migrating at 22 kDa (uncleaved) into a 19-kDa band (cleaved).

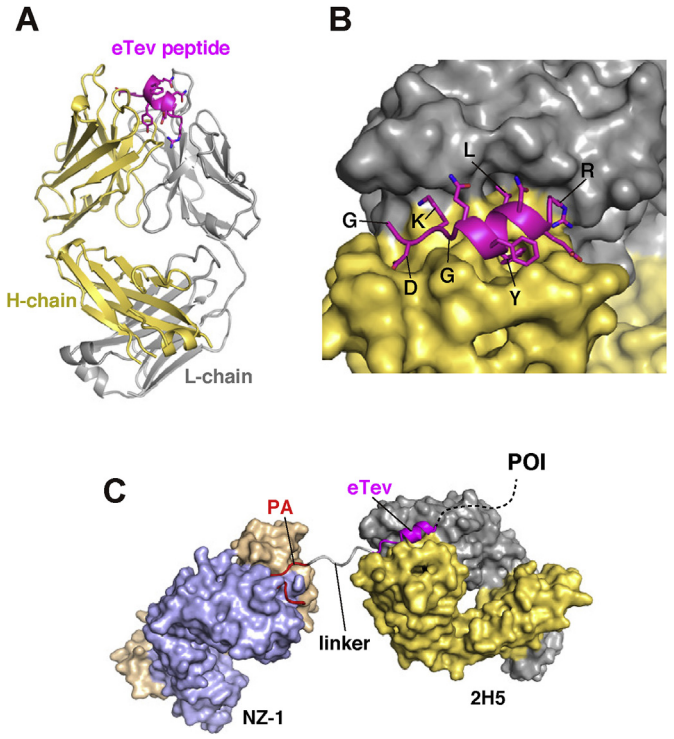

Fig. 3. Structural mechanism of eTev peptide recognition by 2H5. (A) Overall crystal structure of eTev tag peptide bound by 2H5 Fab. Structure is presented in a cartoon model with each chain differently colored in magenta (eTev), gold (2H5 heavy chain), and silver (2H5 light chain). Side chains for the eTev peptide are also shown in stick models. (B) Close-up view of the eTev-2H5 interface. eTev peptide is shown as in (A), and the residues critical for the recognition are labeled. (C) Simulated model of the tandem double tag "cPAW" simultaneously bound by 2H5 and NZ-1 Fab. The model was obtained by manually aligning eTev-2H5 complex structure (this study) and previously determined PA-NZ-1 complex structure (PDB ID:4YO0) side-by-side and the intervening heptapeptide segment (GGGGSHM) was arbitrarily modeled as extended peptide. Note that a turn-forming PA tag peptide (red) is bound at the interface between heavy (lightblue) and light (wheat) chains of NZ-1 Fab with the C-terminus exposed to solvent. The N-terminus of the cPAW would be fused to a protein of interest (POI) through a short linker segment (dotted line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

E2, N3, F6, and Q7 did not have any effect, suggesting that the side chains of these residues do not contribute to the binding. Ala substitution at M0, which was not present in the immunized peptide, did not have any effect, as expected. To our surprise, Ala substitution at G11 in the context of eTev-Fn significantly attenuated the binding, even though the immunized peptide had Cys at this position which was used for the conjugation with KLH. Although the mechanism of the acquisition of G11 specificity by 2H5 is unclear, the above result defined the epitope structure required for the full reactivity with 2H5 as an 11-residue segment containing the following sequence: RxxLYxxGKDG. When we analyzed the susceptibility of each Ala mutant to the TEV protease cleavage, L4, Y5, and Q7 were found to be important for the recognition by the enzyme (Fig. 2B). This result is in general agreement with the TEV substrate specificity reported earlier [8] but is clearly different from the recognition specificity of the 2H5 antibody shown in Fig. 2A, suggesting that the recognition mode for the antibody and the enzyme are different. Collectively, we succeeded in isolating a monoclonal antibody recognizing an 11-residue peptide including the TEV protease recognition motif with a modest solution affinity.

2.2. Crystal structure of 2H5-eTev complex

In order to know the detail of the antigen recognition mechanism of 2H5 antibody, we crystallized the Fab fragment of 2H5 in complex with the eTev peptide. The structure determined at 2.05 Å resolution revealed that the eTev peptide forms nearly 2-turns of α-helix in the binding pocket between the H and L chains of 2H5 Fab (Fig. 3A). All of the 11 residues can be clearly assigned in the electron density, and the entire peptide was accommodated in the deep groove formed by the CDR loops of both chains (Fig. 3B). The helical conformation of the bound eTev peptide explains why the recognition motif spans as long as 11-residue portion, with critical amino acids appearing in a periodical fashion. In fact, all the residues determined as critical for the interaction by the alanine scanning experiments (i.e., R1, L4, Y5, G8, K9, and D10) are in direct contact with the antibody, while side chains of the non-critical residues are all pointing away from the 2H5 surface (Fig. 3B). Although the N-terminus of the eTev peptide is well exposed above the pocket and pointing outward, C-terminal end (residue 11) is located at the bottom of the deep binding groove near the exit point, explaining why glycine is preferred at this position. Nevertheless, both ends of the binding groove are open to the solution, suggesting that eTev tag can be placed in between two other components, namely, a protein of interest and a second epitope tag.

2.3. Design of tandem double tag

If we are to use eTev tag in a “tandem double tagging” format compatible with a sandwich ELISA, 2H5 and the other anti-tag antibody have to bind to a stretch of peptide sequence simultaneously without the steric occlusion by each other. Based on the structure of eTev-2H5 complex described above, we predict that eTev tag and a neighboring recognition sequence must be intervened by at least 6 (for N-terminal side) or 7 (for C-terminal side) residues of flexible linker. As the second

functional unit that constitute tandem double tag along with eTev, we chose PA tag that had been developed in our group [9]. PA tag is a dodecapeptide epitope tag with a sequence of GVAMPGAEDDVV, and is recognized by an antibody NZ-1 with an extremely high affinity. For an N-terminal tagging, PA tag was followed by eTev tag with an intervening 6 residue linker, resulting in a 29-residue "nPAW" tag (GVAMPGAEDDVV*GGGGNS*RENLYFQGKDG). For a C-terminal tagging, PA tag was preceded by eTev sequence with 7-residue linker, resulting in a 30-residue "cPAW" tag (RENLYFQGKDG*GGGGSHM*GVAMPGAEDDVV). Using the crystal structure of PA tag complexed with NZ-1 Fab that has been determined in our group [18], we can simulate binding of both 2H5 and NZ-1 antibodies to the neighboring motifs presented in the context of either cPAW or nPAW tag. Resultant structural model revealed that the linker between PA and eTEV portions is predicted to be long enough to ensure wide separation of the two binding sites that allows simultaneous binding of 2H5 and NZ-1 in cPAW (Fig. 3C) as well as in nPAW (data not shown).

2.4. Sandwich ELISA

The compatibility of the tandem double tag with the sandwich ELISA detection was assessed by using a model protein T4 lysozyme (T4L). To this end, nPAW-T4L and T4L-cPAW are constructed to contain N-terminal His tag and expressed in *E. coli* cytoplasm (Fig. 4A). They were purified using Ni-NTA resin, and subjected to a sandwich ELISA system where NZ-1 and 2H5 were used as capturing and detection antibodies, respectively (Fig. 4B). As shown in Fig. 4C, both nPAW-T4L and T4L-cPAW in the test solution could be detected at concentration as low as 0.5 nM (12.5 ng/ml), suggesting a full compatibility of these tags in this sandwich ELISA. More importantly, both tagged versions produced similar dose-dependent curves saturating at > 10 nM. It is clear from this result that the two antibodies can indeed bind to a single polypeptide without mutual competition, regardless of the order of the two tags in the linear sequence. We next went on to test if the tandem double tagging can be applied to larger and more complex proteins. To this end, cPAW tag was appended to human serum albumin (HSA, 70 kDa) or mouse neuropilin-1 ectodomain (Nrp-1ec, 95 kDa) (Fig. 4A). These proteins were transiently expressed in HEK293 cells, purified from the culture supernatants to homogeneity, and subjected to the sandwich ELISA as above. In spite of the large difference in their molecular property (size, multi-modular nature, and the presence of glycan chains), all three exhibited quite similar concentration-dependency on molar basis (Fig. 4D). This result not only confirmed the universal applicability of the "tandem double tag" to a wide range of target molecules, but also suggested a possibility of using it for a quantitative purpose in a standardized assay format.

2.5. Application to a rapid screening of stable cell clone producing tagged protein

Preparation of high-quality glycoprotein sample for structural analysis often demands establishment of stable cell line due to the low production rate of mammalian cells as well as the low yield during the purification. As a "high producer cell line" can be obtained by chance, it is important to perform large-scale screening of the transfected cells for the expression level of the target protein. We applied the tandem double tag strategy to our effort to isolate stable cells expressing mouse Wise protein. Wise (also known as USAG-1, Sostdc1, Ectodin) is an ~190-residue secreted glycoprotein known to antagonize BMP and Wnt signaling pathway [19]. After stably transfecting Expi293F cells with nPAW-Wise, it was possible to screen a large number of cell clones by using the sandwich ELISA system established above in just one experiment (Fig. 5A). From the 21 cell clones shown highly positive in the first screening (shown red in Fig. 5A), four cell clones exhibited rapid growth and sustained high expression of Wise (Fig. 5B). Finally, we chose clone #29 for the large scale culture and protein production,

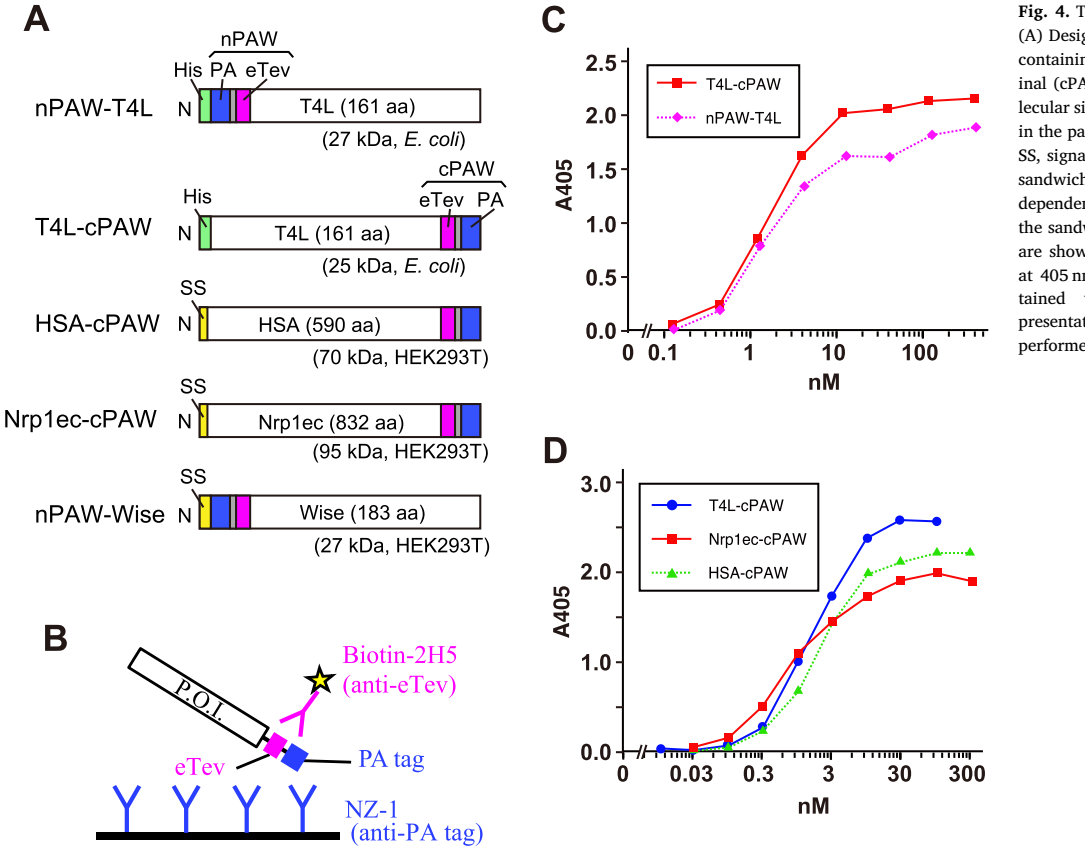

Fig. 4. Tandem double tag and sandwich ELISA. (A) Design of five different expression constructs containing either N-terminal (nPAW) or C-terminal (cPAW) tandem double tag. Theoretical molecular size for each recombinant protein is shown in the parenthesis along with the expression host. SS, signal sequence. (B) Schematic outline of the sandwich ELISA procedure. (C,D) Concentration dependency of various PAW-tagged proteins in the sandwich ELISA system shown in (B). Signals are shown as endpoint (T = 40 min) absorbance at 405 nm after the subtraction of the value obtained with blank wells. Results are representative of three independent experiments performed in duplicate.

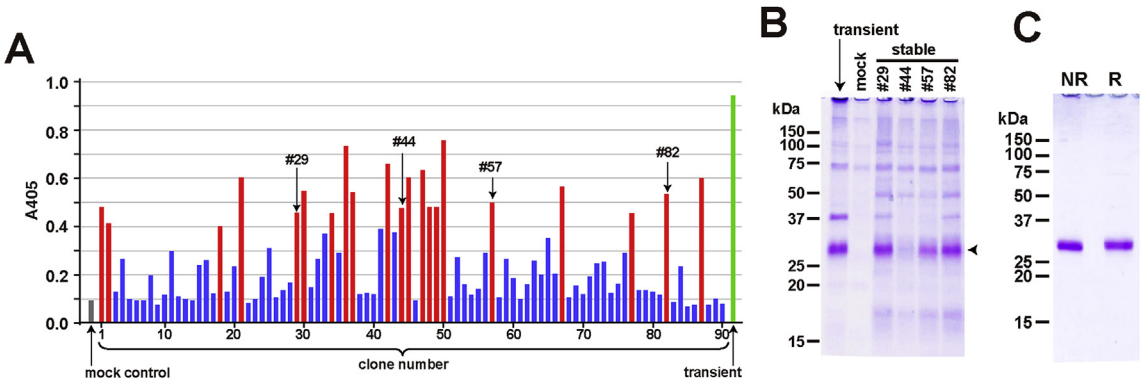

Fig. 5. Rapid establishment of stable cell line producing mouse Wise protein. (A) Culture supernatants from the single-colony wells were screened for the presence of nPAW-Wise using the sandwich ELISA. Out of 90 clones, 21 were considered positive (showing > 4-fold absorbance value compared with the mock control, red bars) and four clones indicated by arrows were chosen for further consideration based on their growth fitness. (B) Confluent culture supernatants from the four stable clones as well as the 4-day old culture media from the transient transfection were subjected to NZ-1 pull-down. Shown is a 15% polyacrylamide SDS-PAGE gel run under nonreducing condition to evaluate the presence of PA-tagged Wise protein (arrowhead). (C) Purity of the final Wise preparation obtained from the cell clone #29. Samples (1 μg/lane) were analyzed by SDS-PAGE using 15% gel under nonreducing (NR) and reducing (R) conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

resulting in a successful preparation of highly pure recombinant Wise protein at an yield of ~1 mg/l culture medium (Fig. 5C).

3. Conclusions

In a recombinant production and subsequent purification of biologically important proteins, availability of antibody-based detection and isolation tools are vital for the success of the project. When the target of the investigation is a membrane protein expressed on cell surface, having one good antibody, either against the protein itself or the genetically fused tag portion, is sufficient for rapid detection and (semi-) quantitation by the use of FACS analysis. For soluble proteins, however, more than one antibody is required to perform quantitative analysis by using sandwich ELISA or like methods. The dual-function eTev peptide reported here can be used as detection and purification tag when simply attached to a protein of interest (POI), but it is most useful when combined with another epitope tag, as represented by the successful creation of “tandem double tag” (Fig. 4). We succeeded in placing eTev and PA tags in a stretch of ~30-residue peptide using the three dimensional structure information about the tag peptide-antibody complex as a guide. When singly attached to N- or C-terminus of POI, the nPAW or cPAW tags enables easy detection and (semi)quantification of the protein through a generalized sandwich ELISA protocol. We predict that this will greatly facilitate large-scale and efficient screening of stable cell clones that secrete high level of valuable proteins for which no good antibodies are available. The hybridoma cell line 2H5 will be deposited in public cell bank for the distribution upon request.

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