Protein Expression and Purification 95 (2014) 240–247

Contents lists available at ScienceDirect

Protein Expression and Purification

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

PA tag: A versatile protein tagging system using a super high affinity antibody against a dodecapeptide derived from human podoplanin

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ARTICLE INFO

Article history:
Received 6 December 2013
and in revised form 20 January 2014
Available online 28 January 2014

Keywords:
Purification method
Immunoaffinity chromatography
Monoclonal antibody
Podoplanin
Peptide tag
Immunodetection
Mammalian cell expression

ABSTRACT

Peptide-based epitope tagging technology is universally used in nearly all kind of research projects that involve biochemical characterization of a target protein, but not many systems are fully compatible with purification purpose. By utilizing an anti-human podoplanin antibody NZ-1, we constructed a novel epitope tag system. NZ-1 possesses exceptionally high affinity toward a dodecapeptide dubbed "PA tag", with a characteristic slow dissociation kinetics. Because of its high affinity, PA-tagged proteins in a dilute sample can be captured by immobilized NZ-1 resin in a near complete fashion and eluted by a solution of free PA peptide. This enabled efficient one-step purification of various proteins including soluble (an ectodomain fragment of neuropilin-1) and membrane (epidermal growth factor receptor) proteins expressed in mammalian cells. Mild regeneration condition of the peptide-bound antibody ensures repeated use of the antibody resin, indicating a cost-efficient nature of the system. Together with its outstanding performance in the immunodetection experiments (i.e., Western blotting and flow cytometry), PA tag/NZ-1 system will offer a great chance to facilitate protein production in many biomedical research projects.

Introduction

Protein purification step represents a serious bottleneck in a basic research as well as industrial applications where high-quality pure protein sample is required. Efficient purification of a recombinant protein is generally achieved by affinity chromatography using genetically fused purification handle or "tag". Among numerous gene-fusion technologies, peptide tag systems have several advantages over "protein tag" systems such as GST-fusion. First, they are less likely to affect structure and function of the target proteins due to the small size (typically 1–2 kDa). For the same reason, it is often not necessary to remove the tag portion after the purification. Another advantage is that there are wide variety of peptide tag systems available we can choose from, including His [1], FLAG [2], HA [3], Myc [4], TARGET [5], Softags [6] and many others, unlike relatively short list of protein tags. Finally, peptide fusion is compatible with virtually all expression hosts and can be expressed either extracellularly or intracellularly. However, peptide-based tagging often suffers from the low specificity and/or affinity. For example, purification of oligohistidine-tagged proteins using metal chelate affinity resin usually results in co-purification of metal-binding proteins present in the starting material, necessitating further purification steps [7]. Epitope tag systems that utilize peptide tag and anti-peptide monoclonal antibody (mAb)[1] generally show high specificity, but even the most popular system such as FLAG tag/anti-FLAG M2 antibody [8] sometimes suffers from non-specific binding to endogenous proteins in certain cell types [9,10]. Another aspect of the tagging system that should be considered is the elution and regeneration condition for the affinity chromatography. Many epitope tag systems are compatible with the mild competitive elution of the tagged protein by free antigen peptide. However, high affinity peptide–antibody interaction is difficult to disrupt and requires high concentration peptide for the elution and harsh regeneration process after each run, resulting in the limited reusability of the affinity resin. To overcome such difficulty, "polyol-responsive" antibodies have been developed where bound antigens can be eluted by solutions containing non-denaturing polyols, eliminating the need for harsh elution/regeneration process [6,11]. However, there are limited number of polyol-responsive anti-tag antibodies available and it is impossible to "grant" such property to a given antibody. Therefore, there is a continuous need for the

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[1] *Abbreviations used:* GFP, green fluorescent protein; mAb, monoclonal antibody; SPR, surface plasmon resonance; TBS, Tris-buffered saline; HEK, human embryonic kidney.

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http://dx.doi.org/10.1016/j.pep.2014.01.009

development of new affinity tag system with high affinity, high specificity, and high reusability.

We have established a unique rat monoclonal antibody NZ-1 directed against 14-residue peptide segment in human podoplanin PLAG domain [12]. Podoplanin is a type I transmembrane protein highly expressed on malignant cancer cells and is implicated in tumor-induced platelet aggregation by binding to CLEC-2 on platelet surface [13]. NZ-1 inhibits binding of podoplanin to CLEC-2 and is a potential lead for the antibody-based therapy against glioblastoma [14]. During the above investigation, we realized that NZ-1 possesses desirable character as an anti-tag antibody. Here, we report a development of a novel affinity tag system consisting of NZ-1 and its epitope peptide dubbed "PA tag". PA tag system proves to outperform many existing affinity tag systems owing to its high affinity, high selectivity, and extended reusability.

Materials and methods

Protein expression

Expression constructs for the T4 lysozyme (T4L) attached with various tags shown in the Supplementary Fig. 1 (PA10-T4L, PA12-T4L, PA14-T4L, T4L-PA10, T4L-PA12, T4L-PA14, T4L-FLAG, T4L-HA and T4L-Myc) were prepared by extension PCR and cloned into *Nco*I-*Bam*HI site of pET16b vector (Novagen). All constructs contained either C-terminal His×8 or N-terminal His×10 tags to facilitate protein purification. Single-residue alanine substitution mutants (G1A, V2A, M4A, P5A, G6A, E8A, D9A, D10A, V11A and V12A) of T4L-PA12 were produced using QuikChange strategy (Agilent). Construct for GFP_{UV}-PA12 was prepared by replacing T4L segment of T4L-PA12 with the coding sequence of GFP_{UV} (Clontech). Full-length DNA for human isocitrate dehydrogenase 1 (IDH1) was attached with tandem FLAG and PA12 tags at the C-terminus and cloned into pcDNA3.1 vector (Life Technologies Corp.) as described previously [15]. For the construction of $mNrp1_{ec}$-PA12, DNA encoding the entire ectodomain of mouse neuropilin-1 (a gift from A. Kumanogoh) and the linker + PA12 segment from T4L-PA12 were fused by extension PCR and cloned into pcDNA3.1 vector. DNA sequence coding for C-terminally truncated construct of human epidermal growth factor receptor (EGFR), which included amino acids 25–1022, was fused with the PA12 tag at the N-terminus and cloned into the pcDNA3.1 vector. Three cysteine residues present in the cytosolic kinase domain (Cys-775, -781 and -797) were replaced with serine using the QuikChange strategy. For the construction of full-length rat neuropilin-2 tagged with PA12 tag, rNrp2-mCherry in pSecTag2A vector (a gift from Y. Goshima) was used to insert PA12 + linker segment (taken from the PA12-T4L) after the signal sequence. All constructs were verified by DNA sequencing.

The tagged T4L proteins were expressed in transformed *Escherichia coli* BL21 (DE3) cultured for ~3 h at 37 °C in the presence of 1 mM IPTG. The T4L proteins were purified from the soluble fraction of the bacterial lysate by Ni–NTA chromatography and dialyzed against phosphate-buffered saline (PBS), pH 6.0. Expression of GFP_{UV}-PA12 was similarly conducted except that the transformed bacteria were cultured for ~24 h at 16 °C in the presence of 1 mM IPTG.

Antibodies used

The rat anti-PA monoclonal antibody NZ-1 (IgG_{2a}, λ) was described previously [12]. The mouse anti-FLAG monoclonal antibody M2, the HRP-conjugated rabbit anti-rat IgG polyclonal antibody, and the HRP-conjugated goat anti-mouse IgG polyclonal antibody were obtained from Sigma–Aldrich. The mouse anti-HA monoclonal antibody 4B2 and the mouse anti-Myc monoclonal antibody 9E10 were obtained from Wako Pure Chemical Industries Ltd. The FITC-conjugated goat anti-rat IgG polyclonal antibody was obtained from Beckman Coulter Inc.

Bio-layer interferometry (BLI)

Binding kinetics of various antibodies toward T4L protein tagged with their respective antigen peptide was analyzed by BLI using Octet RED system (Pall ForteBio). Binding assays were performed in 96-well microtiter plates at 30 °C with orbital sensor agitation at 1000 rpm. Amine Reactive (AR2G) sensors were immobilized with each antibody dissolved at 10–20 μg/ml in 10 mM sodium acetate buffer, pH 6.0, followed by quenching with 1 M ethanolamine, pH 8.5. Tagged proteins were serially diluted in a running buffer (PBS containing 0.005% Tween 20 and 1% BSA) and placed in different wells at a volume of 200 μl. Binding was monitored by dipping the sensors into the wells for 60 s, followed by dissociation in the running buffer for 120 s. Binding curves were analyzed by BIA evaluation software (GE Healthcare) with a curve-fitting using a 1:1 binding model.

Binding kinetics analysis using surface plasmon resonance (SPR)

NZ-1 was immobilized on the CM5 sensor chip using amino coupling chemistry according to the method provided by the manufacturer. The PA tagged T4L proteins were diluted in PBST (PBS (pH 6.0) containing 0.005% Tween 20) and injected at a flow rate of 20 μl/min. For scouting the condition for the mild dissociation of antigen, the NZ-1 surface was first injected with a 100 nM solution of T4L-PA12 to saturate binding sites, incubated with PBST for 200 s to allow free dissociation, followed by an injection of various buffers for 30 s (Supplementary Fig. 2). Dissociation efficiencies were calculated by the ratio of the binding signal (in RU) before and after the injection of each solution (Table 1). All SPR experiments were performed using BIACORE 2000 instrument (GE Healthcare).

Western blot analysis

Human osteosarcoma U-2 OS cells (obtained from the American Type Culture Collection (ATCC), Manassas, VA) were transiently

Table 1
Effect of various buffers on the interaction between PA tag and NZ-1.

Salt, additive	pH (buffer)	Dissociation efficiency (%)
0.15 M NaCl	7.5 (Tris)	0
No	2.0 (Gly–HCl)	91
0.15 M NaCl + 40% propylene glycol	7.5 (Tris)	8
2 M NaCl	7.0 (PIPES)	28
	6.0 (MES)	30
	5.0 (Acetate)	40
	4.0 (Acetate)	87
2 M NaBr	7.0 (PIPES)	52
	6.0 (MES)	59
	5.0 (Acetate)	77
	4.0 (Acetate)	97*
2 M $MgCl_2$	7.0 (PIPES)	93
	6.0 (MES)	94
	5.0 (Acetate)	96*
	4.0 (Acetate)	98*
3 M $MgCl_2$	6.0 (MES)	98*

* Conditions suitable for antigen elution.

transfected with IDH1-FLAG-PA12 and the cell lysate was prepared as described previously [15]. The lysate was separated on 10% SDS–PAGE and transferred to a PVDF membrane, followed by blocking with 4% skim milk in PBS (pH 7.4) with 0.05% Tween 20 for 15 min. For the primary antibody step, the membranes were incubated with varying concentrations of NZ-1 (0.01, 0.1, 1, or 3.5 μg/ml) or 3.5 μg/ml anti-FLAG M2 diluted in Tris-buffered saline, pH 8.0, containing 0.05% Tween 20 (TBST), for 1–60 min. For the secondary antibody step, membranes were incubated for 30 min with either HRP-conjugated rabbit anti-rat polyclonal antibody or HRP-conjugated goat anti-mouse polyclonal antibody (both from Sigma–Aldrich) diluted at 1:6000 in TBST. Four consecutive washes (TBST, 5 min) were conducted between each step. The membranes were developed with ECL™ Prime reagent (GE Healthcare) and analyzed using ImageQuant LAS 4000mini (GE Healthcare).

Flow cytometry

CHO-K1 cells (obtained from ATCC) were transiently transfected with PA12-rNrp2-mCherry construct. After 48 h, the cells were detached, washed, and resuspended in DMEM with 5% FCS (DMEM/FCS). The cells were then incubated with DMEM/FCS containing NZ-1 at various concentrations (0.01, 0.1, 1, or 10 μg/ml) on ice for 30 min, washed twice with DMEM/FCS, and resuspended in DMEM/FCS containing FITC-conjugated goat anti-rat IgG antisera (Beckman Coulter Inc.) diluted at 1:100. After 30 min incubation on ice, the cells were washed once and suspended in PBS (pH 7.4), and analyzed on a flow cytometer (Guava EasyCyte, Millipore).

Purification of PA-tagged proteins

Coupling of NZ-1 to CNBr-activated Sepharose 4 Fast Flow (GE Healthcare) was performed according to the protocol provided by the manufacturer and routinely yielded coupling levels of ~2 mg IgG/ml gel. For the purification of soluble $mNrp1_{ec}$-PA12 secreted from mammalian cells, HEK293T (obtained from ATCC) cells plated in a 15-cm culture dish were transiently transfected with the plasmid and ~25 ml of culture supernatant was harvested ~72 h after the transfection. Cleared supernatant was mixed with NZ-1-Sepharose (250 μl bed volume) and incubated for ~2 h at 4 °C under gentle agitation. The beads were then transferred to a small column, washed with 5 ml of TBS, and the bound protein was eluted with TBS containing 0.1 mg/ml epitope peptide (EGGVAMPGAEDDVV). The peptide used in the elution was synthesized by standard solid-phase synthesis and purified by reverse-phase HPLC (Scrum Inc.). The cost of the peptide solution for the elution would be in the order of ~$100/100 ml, when using a typical peptide synthesis service. The elution was conducted at room temperature in a step-wise manner (250 μl × 10), where dissociation time of 5 min was given for each elution step. For the purification of EGFR from cell membrane, HEK293S GnT1-cells [16] plated in two 15-cm culture dishes were transiently transfected with the plasmid and harvested ~48 h after the transfection. Cells were detached from the plates using PBS containing 0.5 mM EDTA. The pelleted cells were resuspended in 5 ml of a solution containing 20 mM Tris–Cl (pH 8.0), 400 mM NaCl, 10% glycerol and 1 mM EDTA and solubilized by adding 5 ml of the same solution containing 0.4% (w/v) Triton X-100. The solubilized cell lysates were incubated at 4 °C for ~1 h with gentle mixing and further ultracentrifuged 30 min at 50,000×*g* at 4 °C. The cleared lysates were mixed with NZ-1-Sepharose (100 μl bed volume) and incubated at 4 °C for ~2 h under gentle agitation. The beads were washed in a small column with 2 ml of a solution containing 20 mM Tris–Cl (pH 8.0), 400 mM NaCl, 10% glycerol, 1 mM EDTA and 0.1% Triton X-100 and eluted with the same solution containing 0.1 mg/ml epitope peptide in a step-wise manner (100 μl × 10) as described above.

Results and discussion

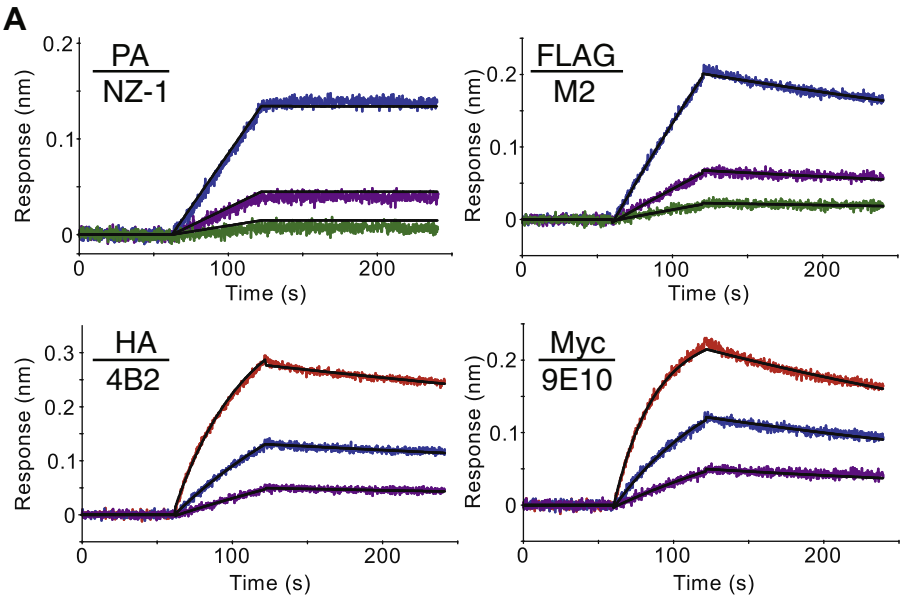
Kinetic analysis of NZ-1/PA tag interaction

Previous investigations have indicated that NZ-1 possesses very high affinity toward podoplanin. Because successful anti-tag antibody system requires the high affinity interaction, we investigated binding kinetics between NZ-1 and its antigenic peptide and compared it with three other tag/mAb pairs that are commercially available as purification systems. Antibodies used were M2 (anti-FLAG, available from Sigma–Aldrich) [17], 4B2 (anti-HA, available from WAKO Pure Chemical Industries Ltd.), and 9E10 (anti-Myc, available from WAKO Pure Chemical Industries Ltd.) [18]. In order to make fair comparison, all peptide tags were fused C-terminally to the same model protein T4 lysozyme (T4L) using identical length of linker (Supplementary Fig. 1). Although all anti-tag mAbs exhibited reasonably good binding behavior toward each matched antigenic peptide in the biolayer interferography experiments using Octet devise (Fig. 1A), NZ-1/PA tag interaction was exceptional in that it did not show dissociation over the assay time of few minutes. In fact, curve fitting of each data set showed exceptionally slow dissociation rate constant for NZ-1/PA interaction, resulting in much higher overall affinity than other tag systems (Fig. 1B). The very slow dissociation is a property highly desirable for isolation purpose, because it assures that tagged protein are completely captured and does not leak during the washing steps.

Minimal sequence requirement for the PA tag

NZ-1 was raised against 14-residue peptide (EGGVAMPGAE DDVV; PA14) corresponding to residues 38–51 of human podoplanin [12]. Previous study showed that the central 10-residue portion (GVAMPGAEDD; PA10) was indispensable for the recognition [19]. In order to determine the minimal peptide region that warrants observed high affinity, we performed surface plasmon resonance (SPR) binding experiments between NZ-1 and various versions of PA tagged protein. As shown in Fig. 2A and B, either PA14 or PA12 (GVAMPGAEDDVV) tags attached C-terminally to T4L bound to NZ-1 with high affinity in an indistinguishable manner, indicating that the N-terminal two residues (Glu-Gly) were dispensable. In contrast, PA10-tagged version showed greatly reduced affinity (Fig. 2C), suggesting that the last two valines were important to ensure the high affinity. We next tested whether PA tag was compatible with N-terminal fusion. As shown in Fig. 2D and E, both PA14 and PA12 mediated high affinity binding to NZ-1 when appended at N-terminus of T4L, although the dissociation was slightly faster than the C-terminal versions. Interestingly, affinity of PA10-T4L was decreased by only 1.5-fold from PA12 and PA14. It seems likely that the presence of the last two residues is important for the high affinity but they can be substituted by other amino acids (such as Arg–Glu in PA10-T4L). Hereafter, we will use PA12 sequence for all applications.

To determine which amino acid residues within the PA12 are critical for the recognition by NZ-1, ten non-alanine residues were mutated to alanine and each mutant (in the format of T4L-PA12) was evaluated in SPR assay. As clearly shown in the overlaid sensorgrams in Fig. 3, mutations at M4 and D10 had the most severe effect, indicating the major contribution of these residues in the binding. Effects of four other mutations (P5A, G6A, E8A, and D9A) were less pronounced, and the rest of the mutants exhibited virtually identical binding behavior to the original PA tag. Therefore, NZ-1 seems to achieve the very high affinity toward PA tag using



B

Tag(Antibody)	k_a (1/M•s)	k_d (1/s)	K_D(M)	χ^2
PA(NZ-1)	1.4×10^{4}	5.8×10^{-6}	4.0×10^{-10}	3.4×10^{-5}
FLAG (M2)	6.1×10^{4}	1.7×10^{-3}	2.8×10^{-8}	1.2×10^{-5}
HA (4B2)	6.7×10^{5}	1.1×10^{-3}	1.6×10^{-9}	1.4×10^{-5}
Myc (9E10)	1.1×10^{6}	2.5×10^{-3}	2.2×10^{-9}	1.3×10^{-5}

Fig. 1. Bio-layer interferometry kinetic analysis of various anti-epitope tag antibodies. (A) Representative BLI binding data for C-terminally tagged T4L proteins (T4L-PA12, T4L-FLAG, T4L-HA, T4L-Myc). Each anti-tag mAb was immobilized on sensor tips. Association (from 60 s to 120 s) and dissociation (from 120 s to 240 s) phases are shown for serial dilution series of the tagged T4L (red, 30 nM; blue, 10 nM; purple, 3 nM, green, 1 nM). Black lines represent a global fit of the data to a 1:1 binding model. (B) Kinetic binding parameters for each interaction obtained by the global fit shown in (A). Successful curve fitting to the model was indicated by the very low χ^2 values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

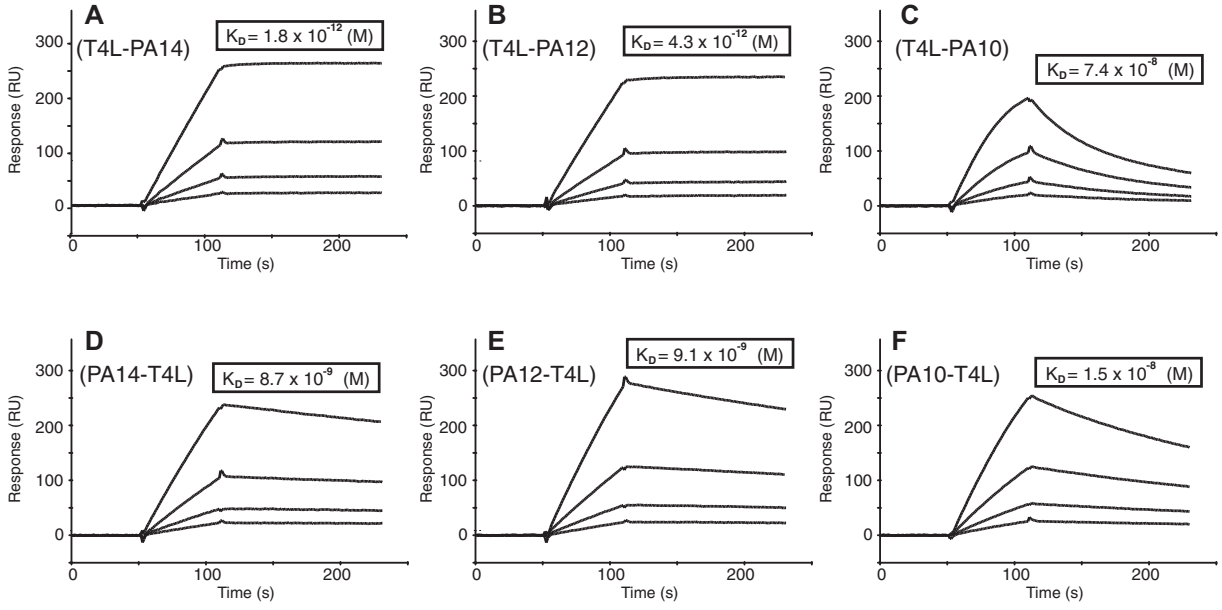


Fig. 2. Surface plasmon resonance kinetic analysis of NZ-1 binding toward various PA tags. Serially diluted PA-tagged T4L proteins (3.125, 6.25, 12.5 and 25 nM) were injected over the CM5 sensor chip immobilized with NZ-1 for 60 s, followed by dissociation in PBST for 120 s at a flow rate of 20 μl/min. PA14 (A and D), PA12 (B and E), or PA10 (C and F) peptides were fused either C-terminally (A–C) or N-terminally (D–F) to T4L protein. Each set of binding curves was globally fitted to 1:1 binding model to derive dissociation equilibrium constant (K_D) values shown in the boxes.

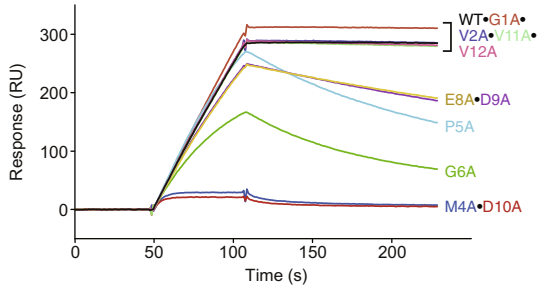


Fig. 3. Effect of alanine substitutions of PA tag sequence on the binding to NZ-1 evaluated using SPR. Purified alanine-substituted versions of T4L-PA12 diluted at 20 nM were injected over the surface immobilized with NZ-1 as described in the legend to Fig. 2 and sensorgrams after subtraction of the buffer control were overlaid.

a combination of many interactions to the central core segment (M4-D10), with the contribution of M4 and D10 being dominant.

Utility of NZ-1 as a detection antibody

The unusually high affinity of NZ-1 toward PA tag made us speculate that it may be used as a mean to detect proteins that are tagged with PA peptide. To test this idea, a cytoplasmic enzyme isocitrate dehydrogenase 1 (IDH1) was doubly tagged with FLAG and PA (Supplementary Fig. 1), and expressed in human osteosarcoma cell line U-2 OS that expresses low endogenous podoplanin [20,21]. As shown in Fig. 4A, Western blotting of the resultant cell lysate with NZ-1 produced a strong band corresponding to the expressed IDH1 (~45 kDa). NZ-1 immunoblot of the untransfected cells did not show any reactive bands (data not shown), indicating very high specificity of the detection. In contrast, numerous non-specific bands other than tagged IDH1 were seen in the anti-FLAG M2 antibody immunoblot (Fig. 4A, middle lane). In addition to the high specificity, detection by NZ-1 was proven highly sensitive, because the signal intensity of the reactive band was saturated after incubating with NZ-1 for as short as 5 min (Fig. 4B), and at concentration as low as 0.1 μg/ml (Fig. 4C).

Next we applied NZ-1/PA tag combination to the flow cytometric analysis of cell surface receptors. An expression construct for a type I transmembrane protein neuropilin (Nrp)-2 was engineered

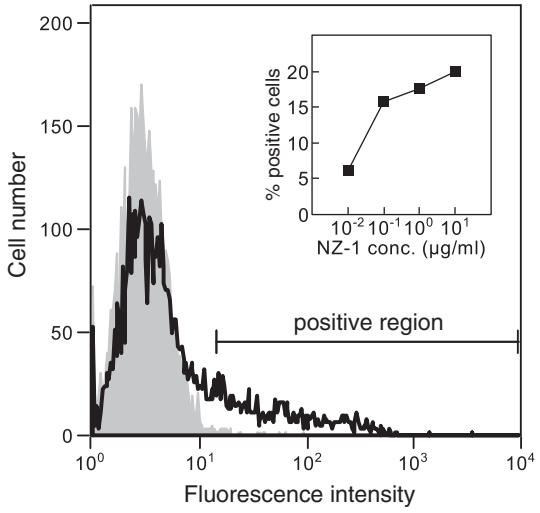


Fig. 5. Flow cytometric analysis of PA-tagged membrane protein. CHO-K1 cells (gray area) or CHO-K1 cells transiently transfected with PA12-rNrp2-mCherry (solid line) were incubated with 10 μg/ml NZ-1 and stained with FITC-labeled secondary antibody specific for rat IgG. Less than 1% of parental CHO cell population was positive with NZ-1 (region indicated by the bracket). Staining efficiency was expressed as the % positive cells and plotted against the concentration of primary antibody (inset).

to carry PA tag at its N-terminus (extracellular domain) and mCherry at its C-terminus (cytoplasmic domain), and was transiently transfected into CHO-K1 cells. Inspection of cells by fluorescence microscopy after transfection indicated that ~20% cells were positive for the Nrp2-mCherry. Similar level of surface expression was confirmed by the flow cytometric analysis of the same cells with NZ-1 (Fig. 5, solid line histogram). Furthermore, efficient staining was achieved even when the concentration of the primary antibody was reduced to 0.1 μg/ml NZ-1 (Fig. 5, inset), again underscoring the great potential of PA tag as a detection system. One potential shortcoming of the NZ-1/PA tag system is the incompatibility with human cell lines expressing endogenous podoplanin. However, the NZ-1-reactive sequence is unique to human podoplanin and both M4 and D10 are absent in mouse, rat, hamster, or dog podoplanin (Supplementary Fig. 3), indicating that cell lines from these animals are compatible with this system. In fact,

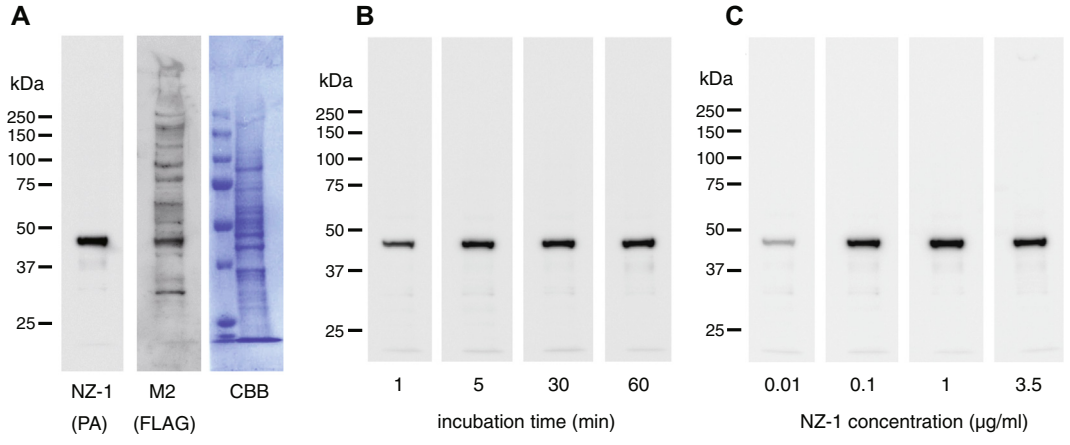


Fig. 4. Western blot analysis of PA tagged IDH1 with NZ-1. (A) Total cell lysates from human osteosarcoma cells expressing the IDH1-FLAG-PA12 tag were electrophoresed under reducing conditions using 10% SDS–PAGE gel and transferred to a membrane. Three strips of the membrane containing the same amount of lysate were immunoblotted with NZ-1 (left), M2 (center), or directly stained with Coomassie Brilliant Blue (right). The same membrane strips were used to assess the time course (B) or dose-dependency (C) of the NZ-1 incubation. In (B), membranes were incubated with 1 μg/ml NZ-1 for the indicated period. In (C), membranes were incubated with varying concentrations of NZ-1 for 30 min. All steps after the NZ-1 incubation, including secondary antibody reaction and chemiluminescence visualization, were conducted under the exactly same condition for all membranes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

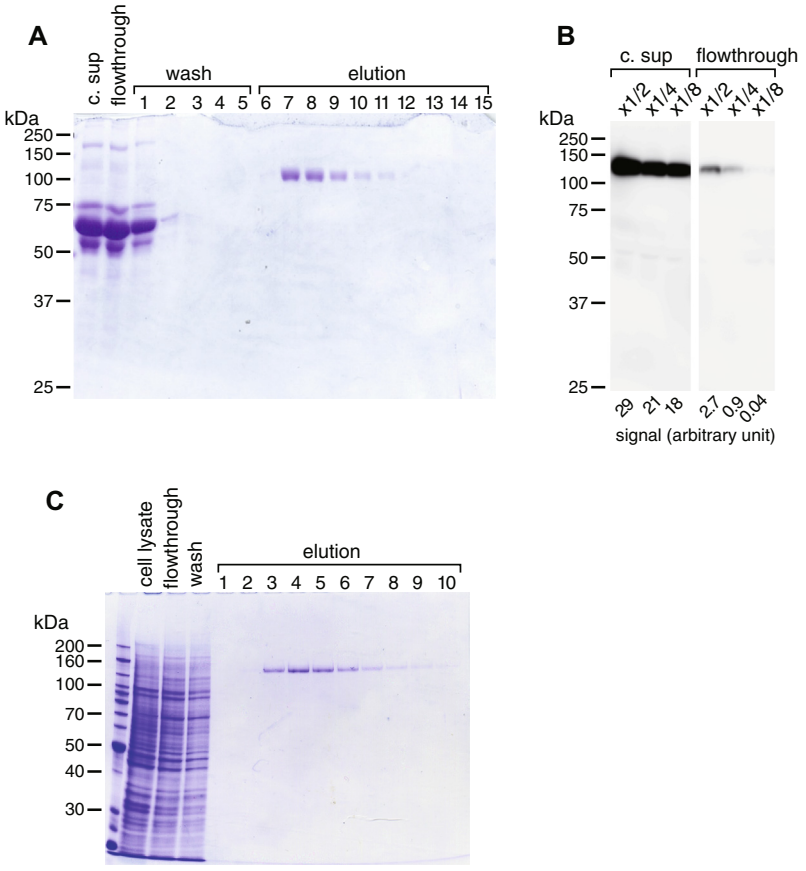


Fig. 6. One-step purification of PA12-tagged proteins. (A) Purification of soluble ectodomain fragment of mouse neuropilin-1. Five microliters of the culture supernatant from the transfected cells before (c. sup) and after (flowthrough) NZ-1-Sepharose capture, as well as 10 ml of the five wash (lanes 1–5) and ten peptide-elution fractions (lanes 6–15) during the column chromatography were subjected to 10% SDS–PAGE under reducing condition and stained with CBB. (B) The culture supernatant and the flowthrough fractions from the chromatography shown in (A) were diluted with TBS as indicated and subjected to Western blotting using NZ-1. Intensity of the 120-kDa band was quantified by densitometric analysis and shown below each lane. (C) One step purification of EGFR from the HEK cells. Ten microliters of the detergent-solubilized cell lysates from transfectants before (cell lysate) and after (flowthrough) NZ-1-Sepharose capture, as well as the 10 ml of the wash and ten peptide-elution fractions during the column chromatography were subjected to 10% SDS–PAGE under reducing condition and stained with CBB.

NZ-1 does not exhibit any binding toward CHO cells in flow cytometry (Fig. 5, shaded histogram) or in Western blotting (data not shown). Furthermore, presence of endogenous podoplanin does not cause problems when the system is used for a purification purpose as will be described in the next section.

Application to the protein purification system

We next explored the possibility to apply NZ-1/PA tag interaction in a protein purification system. First we focused on the purification of recombinant proteins secreted into culture media from transfected mammalian cells, because such project generally suffers from the lack of robust purification method due to the low abundance of the protein in the starting material (i.e., culture supernatants). As shown in Fig. 6A, soluble ectodomain fragment of mouse Nrp1 tagged C-terminally with PA12 could be efficiently captured onto NZ-1-Sepharose from the dilute culture supernatants of the transfected HEK293T cells, and eluted from the resin by a solution containing 0.1 mg/ml free PA14 peptide. The ability of free peptide to competitively elute bound antigen was rather unexpected, because the interaction seemed to be almost irreversible in the kinetic binding assays shown in Figs. 1 and 2. In fact, we found it essential to perform the elution in a step-wise manner rather than under continuous flow, and allow ~5 min of dissociation time for each fraction. The near-perfect capture of tagged protein during the affinity chromatography was confirmed by the presence of less than 5% antigen in the flowthrough fraction (Fig. 6B). Importantly, protein eluted from the resin was extremely pure, and ready for use in the downstream experiments without further purification. We have so far applied PA tag/NZ-1 system to purify more than 15 secreted proteins having widely different molecular weight (from 15 to >300 kDa), where >95% purity was regularly attainable in one step by NZ-1-Sepharose chromatography.

We also tested if PA tag system is compatible with the purification of membrane proteins solubilized with detergents. HEK293S GnT1$^-$ cells were transfected with N-terminally PA-tagged human EGFR, and the harvested transfectants were solubilized with 0.2% Triton X-100. As clearly shown in Fig. 6C, PA-tagged EGFR was captured by NZ-1-Sepharose and eluted efficiently with 0.1 mg/ml free PA14 peptide solution. A great separation power of the PA tag/NZ-1 system is demonstrated by the successful purification of the 145-kDa EGFR protein from the low abundance cell lysate, with virtually no contaminations. To our surprise, podoplanin (~37 kDa) was not co-purified with the PA-tagged proteins even though the cell line expresses endogenous human podoplanin. It is possible that the effective affinity of NZ-1 for the epitope region (PLAG domain) of native podoplanin is relatively low, probably due to the steric hindrance of the peptide sequence by the nearby glycan chains [22], resulting in the inefficient capture during the affinity chromatography. In any case, this result indicates that NZ-1/PA tag is fully compatible with membrane protein purification in the presence of detergents, even from the cell lysates containing endogenous human podoplanin.

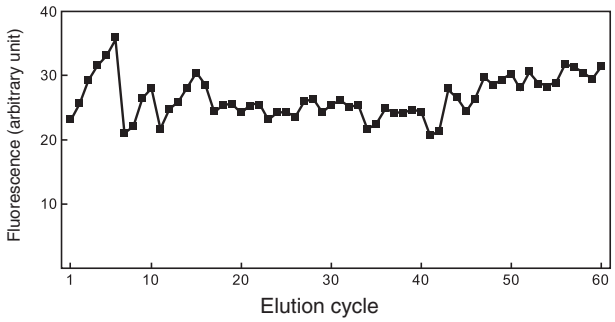


Fig. 7. Effect of the repeated column regeneration treatments on the yield of PA-tagged GFP_{UV} purification by NZ-1-Sepharose. One purification cycle constituted of (i) application of bacterial lysate containing GFP_{UV}-PA12 to a small column of NZ-1 Sepharose, (ii) washing with 4 column volumes of TBS, and (iii) elution/regeneration by adding 5 column volumes of 3 M $MgCl_2$ in 10 mM MES, pH 6.0. The graph shows the amount of GFP_{UV}-PA12 contained in the pooled eluate fraction from each purification cycle quantified with a fluorescence measurement.

Reusability of the NZ-1 affinity resin

In general, antibody-based purification systems tend to be costly compared to other methods that use affinity resins with immobilized chemical compounds [7]. Although the major reason for the high cost is the requirement of the use of synthetic peptide for the elution process, it is often noted that the limited reusability of the antibody resin is another cost-elevating factor. This is especially critical in an immunoaffinity system where the antibody binds its antigen tightly, because potentially damaging condition (e.g., high or low pH, denaturing agents, etc.) is required for the regeneration of the column to remove the peptide that was bound during the elution process. We therefore sought to find conditions that can completely disrupt NZ-1:PA tag interaction while maintaining the functional integrity of the antibody. To this end, NZ-1 was covalently immobilized on a Biacore sensorchip and allowed to saturate with T4L-PA12, followed by an injection of various buffers (Supplementary Fig. 2). Table 1 shows the effect of brief (30 s) exposure to a variety of conditions on the dissociation of the bound antigen. Unlike some "polyol-responsive" antibodies [6,11], inclusion of water-miscible organic solvent such as propylene glycol did not have dissociating effects. Strongly acidic condition (pH 2.0) was effective in disrupting the interaction, but it severely damaged the NZ-1 itself, because the surface lost binding ability (data not shown). Lowering the pH of the buffer was generally effective, especially when high concentration of salt was included. After testing several combinations, we found that a slightly acidic (i.e., pH 6.0) buffer containing 3 M $MgCl_2$ could completely remove bound antigen from the antibody. It was confirmed that brief injection of this buffer onto the NZ-1 surface did not affect the binding capacity.

We adopted the above buffer as a column-regeneration condition after each cycle of protein purification using NZ-1-Sepharose. In order to assess potential damaging effect of the regeneration condition, GFP_{UV}-PA12 was expressed in bacterial cytosol and an aliquot of cell lysate was applied to a small column of NZ-1-Sepharose, followed by direct elution with 3 M $MgCl_2$, pH 6.0. Measurement of the total fluorescent intensity of the cell lysate before and after passing through the column confirmed more than 95% capture of GFP_{UV}-PA12, as well as 100% recovery of the captured protein in the eluted fraction (data not shown). Most importantly, we observed sustained fluorescence recovery when this absorption-elution/regeneration cycles were repeated up to 60 times, indicating that the NZ-1 column could withstand 60 times of regeneration cycles (Fig. 7). It is therefore clear that the NZ-1-Sepharose can be efficiently regenerated by the treatment with 3 M $MgCl_2$ at pH 6.0 without causing any damage on the immobilized antibody. It is important to note that direct elution with 3 M $MgCl_2$ rather than the expensive 0.1 mg/ml peptide solution can be employed during the purification step, when the target protein is known to survive brief exposure to this condition.

Conclusion

We have successfully developed a novel affinity tagging system "PA tag" by applying a unique mAb NZ-1 against human podoplanin. Owing to its unusually high affinity toward linear dodecapeptide antigen, NZ-1 can be used to detect, capture, and purify tagged proteins in various assay formats. The biggest advantage of this system is the ability to purify low abundant proteins at high yield in one step, along with its extended reusability. Major limitation does exist, however, in that it is not fully compatible with the human tissues/cells that express endogenous podoplanin. Among commonly used cell lines, we have confirmed that HEK cells and COS cells are positive for NZ-1 staining. Many but not all cancer cell lines express podoplanin [12,21]. Nevertheless, these cells can be used as expression hosts if the purpose is to purify PA-tagged recombinant proteins, because the contamination of endogenous podoplanin is negligible (see Fig. 6C). On the other hand, PA tag system is universally useful in a wide variety of applications dealing with non-human tissues/cells. Because the two most critical residues in the PA tag for the high affinity recognition by NZ-1 (i.e., M4 and D10) are substituted to very different amino acids in mouse, rat, hamster, and dog podoplanins (Supplementary Fig. 3), cells/tissues from these animals should be fully compatible with the PA tag/NZ-1 system, as exemplified by experiments with CHO-K1 cells (Fig. 5). Furthermore, utility of the PA tag system may not be limited to the applications described in the current study. For example, PA-tagging and NZ-1 capture may be used in a proteomic interactome analyses, because such study requires high affinity (or more accurately, slow dissociation) between the tagged bait and the capture device to withstand extensive washing to eliminate nonspecific binders. Overall, it is anticipated that the PA tag system will be a unique and powerful addition to our list of experimental tools in the field of protein expression and purification.

Acknowledgments

We would like to thank Keiko Tamura-Kawakami, Emiko Mihara, and Rika Oi for their excellent technical support, and Mie Sakai for preparation of the manuscript. This work was supported by the Platform for Drug Discovery, Informatics, and Structural Life Science grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2014.01.009.

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