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Production of a common epitope specific anti-ankyrin monoclonal antibody

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ABSTRACT

Background: Ankyrin (Ank) is a protein family with crucial roles in retaining normal cellular physiology. In addition, ankyrin offers the potential as a non-antibody binder against various biomolecules. The designed ankyrin repeat protein (DARPin) selected from phage display libraries is useful for molecular detection and therapy. Monoclonal antibodies (mAbs) specific to the common epitope of DARPin are required to detect protein-protein interaction.

Objectives: This study aimed to establish mAbs against common antigenic determinant of ankyrins for further application in immunological techniques.

Materials and methods: Ank1D4 monomer and dimer were generated in the *Escherichia coli* expression system for immunogen preparation and validation of established mAbs. The binding activity of anti-Ank mAb obtained from different hybridoma clones was characterized using Ank1D4 by indirect ELISA. Candidate anti-Ank mAbs were validated for their cross-reactivity against irrelevant ankyrin (Ank2D3). The binding kinetic of mAbs from three candidate hybridoma clones (Ank-54, Ank-59, and Ank-94) was evaluated using bio-layer interferometry (BLI). The highest affinity clone (Ank-94 mAb) was further validated for its specificity against Ank1D4 and dimeric Ank1D4 using indirect ELISA. The interaction of three anti-Ank mAbs and ankyrins was compared by western immunoblotting analysis. The specificity of Ank-94 mAb was determined using a closely related scaffold, i.e., alpha-helicoidal HEAT-like repeat protein scaffold (αRep) by indirect ELISA. Ankyrins were detected by sandwich ELISA using Ank-94 mAb.

Results: The culture supernatant from hybridoma clones were characterized for their anti-ankyrin binding properties. Using indirect ELISA, three clones exhibited positive reactivity against the immunized ankyrin antigen (Ank1D4). The interactive epitope was found to rely on common antigenic determinants found in Ank1D4, dimeric Ank1D4, and an irrelevant ankyrin, Ank2D3. The immunoblotting results suggest that all mAbs interact with the sequential epitope of ankyrins. The cross-reactivity of Ank-94 mAb was not observed with αRep. Ank-94 mAb was selected for further purification and evaluation of binding properties due to its highest degree of binding affinity against Ank1D4.

Conclusion: The establishment of a novel Ank-94 mAb could be a valuable research tool in tracing the target of DARPins or developing immunoassays. Ank-94 mAb is superior over formerly produced Ank mAbs since it recognizes a common epitope on DARPins and relies on sequential epitope. Ank-94 mAb has no cross-reactivity with another scaffold, αRep.

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Introduction

Cellular scaffolds, including fibronectin type III domain (FN3) scaffolds, Kunitz domains, and ankyrins, are involved in maintaining the normal physiology of cells. The level of these scaffold proteins has been significantly associated with certain pathogenesis.[1, 2] FN3 scaffold expression, a component of the extracellular matrix, has been observed to decrease lung carcinomas and renal disease incidences.[3] High expressions of Kunitz domains (protease inhibitors) have been implicated in the pathogenesis of chronic pancreatitis and Alzheimer's disease; whereas, a reduced degree of expression was observed in cases of cervical cancer.[4–7] Apart from the extracellular scaffolds, an absence of intracellular-associated ankyrin-R can impact the depletion of protein 4.2, Rh, and RhAG in hereditary spherocytosis patients.[8] To monitor the expression of scaffold levels, monoclonal antibodies specific to individual scaffolds have been developed to be applied in flow cytometry, immunohistochemistry, immunoblotting, or immunocyto-chemistry.[8–10] The monoclonal anti-Kunitz protease inhibitor (KPI) captured with the human beta-Amyloid protein precursor (APP) is useful in the early diagnosis of Alzheimer's Disease detected by sandwich ELISA[5,11] Mouse ankyrin B monoclonal antibody was generated to recognize the spectrin-binding domain, which corresponded to a subset of olfactory receptor neuron (ORN) axons.[9] The monoclonal antibody against ankyrin-R (ankyrin-1) and erythrocyte ankyrin was produced to quantify ankyrin expression via flow cytometry.[8]

Designed ankyrin repeat proteins (DARPins) are small, high-affinity proteins that mediate specific protein-protein interactions.[12,13] The ankyrin structure comprises 33-amino acid residues in which conserved and variable residues are built from stacked. Each repeat forms a structural unit containing a β-turn followed by two antiparallel α-helices and a loop that connects it with the next repeat.[14,15] The structural compatibility of the natural repeat domains consists of a continuous hydrophobic core and a hydrophilic conserved molecular surface, both of which are stabilized from the capping repeats.[16] The distinct capability of ankyrin function involves the membrane-binding domain, which involves binding many unrelated proteins.[17] Various DARPins have been investigated to discover alternative protein therapeutics that rely on variable residues to repeat.[18,19]

We established a novel ankyrin monoclonal antibody (mAb) with broad immunoreactivity against the conserved region in the present study. In previously published reports, Ank1D4 was used to immunize BALB/c mouse for standard hybridoma production. Ank1D4 contains three internal repeats and is flanked by N- and C- terminus caps with certain variable residues that determine the specific recognition of the HIV-1 Gag domain.[13,20] An irrelevant Ank2D313 with different variable residues, along with a newly generated Ank1D4 dimer, was co-examined for the immunological activity and specificity of selected monoclonal antibodies.

Materials and methods

Construction and expression of ankyrin

Recombinant monomeric Ank1D4 and Ank2D3 were constructed and produced according to the previously described method.[13] Dimeric Ank1D4 was subsequently generated by relying on the monomeric Ank1D4 sequence; wherein each module was connected by a flexible $(G_4S)_4$ linker peptide. Accordingly, 6×HIS dimeric Ank1D4 was incorporated into the pQE-30 expression vector (Qiagen, Germany) (Figure 1) and transformed into *Escherichia coli* strain XL1-blue (Stratagene, San Diego, CA, USA) for plasmid propagation. *E. coli* XL1-blue harboring plasmid pQE-30-6×His dimeric Ank1D4 was confirmed using a pair of primers: Fw_NAnk1 (5'-TCC GCG GCC GCA GAC CTG GGT AAG-3') and Rev_Ank23C (5'-GCT AAT TAA GCT TTG CAG GAT TTC AGC-3'). After the purification process with QuickGene Plasmid kit S II (Kurabo, Germany), the plasmids were effectively sequenced (Agentide Inc, USA). In addition, pQE-30-6×His dimeric Ank1D4 was transformed into the *E. coli* strain M15[pREP4] (Qiagen, Germany) for protein expression.

***Figure 1**. Amino acid sequence of ankyrin repeats consists of conserved (black) and variable residues (red), flanking by N- and C- terminal capping repeats.*

Animals and immunization

Two 6-8-week-old BALB/c mice were obtained from M-CLEA Nomura Siam (Bangkok, Thailand). Accordingly, the Animal Care and Use Committee of Chiang Mai University approved all animal experiments. To produce anti-Ank mAb, a standard hybridoma technique was performed. Mice were intraperitoneally immunized with recombinant 6×HIS tagged Ank1D4 (100 µg) using Freund's adjuvant at two-week intervals for three immunizations. Complete Freund's adjuvant was used in the first immunization procedure, and incomplete Freund's adjuvant was used in the second and third immunizations. Seven days after the immunizations

were administered, blood was collected from the immunized mice by superficial temporal vein venipuncture. The presence of a specific antibody was determined by indirect ELISA. The mouse receiving the higher antibody titer was boosted with 6×HIS Ank1D4 (100 µg) in PBS. Five days after this boosting, splenocytes were harvested and fused with Sp2/mIL6 myeloma cells (ATCC, USA) using 50% polyethylene glycol. After cultivation in hypoxanthine, aminopterin, and thymidine (HAT) selective medium (GIBCO, UK), culture supernatants obtained from hybridoma were examined to determine the degree of antibody reactivity through interactions with 6×HIS Ank1D4 using indirect ELISA. The hybridomas that produced the 6×HIS Ank1D4 specific antibody were subjected to single cell cloning with limited dilutions. All mouse anti-ankyrin monoclonal antibodies produced by the clones were isotyped using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science, Germany). For simplicity, anti-ankyrin monoclonal antibodies will be denoted as anti-Ank mAb(s) and Ank - (number of clone) mAb throughout the paper.

Binding activity of anti-Ank mAb

To characterize anti-Ank mAb from different hybridoma clones, indirect ELISA was performed. Ank1D4 (1 µg/mL) was immobilized in a microtiter plate (Greiner, Germany) and left overnight at 4 °C in a moisture chamber. The coated wells were washed four times with PBST (PBS containing 0.05% Tween 20) and were blocked with 2% w/v bovine serum albumin (BSA) and phosphate buffer saline, pH 7.4 (PBS) for 1 hr at RT. After being washed with PBST, Ank1D4 was incubated with the culture supernatant form of mouse anti-Ank mAb (1:100 dilution) for 1 hr at RT. After being washed with PBST, a 1:3,000 dilution of goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP) (KPL, USA) in 2% w/v BSA in PBS was added. The resulting specimen was subsequently incubated for 1 hr at RT. The binding of ankyrin was detected by adding 3, 3', 5, 5'-tetramethylbenzidine (TMB) membrane peroxidase substrate (KPL, USA). Subsequently, the degree of absorbance was monitored at 450 nm.

Examining the specificity of mAbs against irrelevant ankyrin

Indirect ELISA was applied to determine the cross-reactivity of the candidate anti-Ank mAbs against an irrelevant ankyrin, i.e., Ank2D3. Ank1D4 or Ank2D3 (1 µg/mL) was immobilized in a 96-well plate and left overnight at 4 °C in a moisture chamber. The coated wells were washed four times with PBST and blocked with 2% w/v BSA in PBS for 1 hr at RT. Each protein was dissolved in PBS with 2% w/v BSA. After being washed with PBST, ankyrins were incubated with the culture supernatant form of mouse anti-Ank mAb (Ank-54, Ank-59, and Ank-94 mAbs) (1:100 dilution) for 1 hr at RT. After being washed with PBST, a 1:3,000 dilution of goat anti-mouse Igs conjugated with HRP was added. The resulting specimen was incubated for 1 hr at RT. The binding of anti-Ank mAb was detected by adding a TMB substrate. Subsequently, the degree of absorbance was monitored at 450 nm.

Comparison of binding activity against mono and dimeric ankyrin

The immunoreactivity of a selected mAb clone Ank-94 against monomeric and dimeric Ank1D4 was determined using indirect ELISA. The equimolar of monomeric (0.05 µg/mL) and dimeric (0.1 µg/mL) Ank1D4 was prepared and immobilized in a microtiter plate. The resulting specimen was subsequently incubated at 4 °C overnight. The coated wells were washed four times with PBST and incubated in PBS with 2% w/v BSA for 1 hr at RT. Each protein was dissolved in 2% w/v BSA in PBS. After being washed with PBST, ankyrins were incubated with a culture supernatant form of Ank-94 mAb (1:200 dilution) for 1 hr at RT. After being washed with PBST, a 1:3,000 dilution of goat anti-mouse Igs conjugate was added, and the resulting specimen was subsequently incubated for 1 hr at RT. The binding of ankyrin was detected by adding a TMB substrate. The degree of absorbance was then monitored at 450 nm.

Western immunoblotting analysis against ankyrins

His-tagged ankyrin proteins were purified from *E. coli* strain M15[pREP4] using affinity chromatography on an HisTrap column with ÄKTA pure™ (Cytiva, Germany). Recombinant Ank2D3, monomeric Ank1D4, and dimeric Ank1D4 were separated by 15% SDS-PAGE and were subjected to immunoblotting analysis. After treatment with a blocking solution (2% w/v skim milk in PBS), the nitrocellulose membranes (Cytiva, Germany) were incubated with a specific blocking solution containing mouse anti-Ank mAb. Ank-54, Ank-59, or Ank-94 mAbs at 1:3000 dilution for 1 hr followed by goat anti-mouse Igs conjugated HRP (1:3000 dilution). The membrane was treated with an enhanced chemiluminescent reagent (Rockford, IL, USA). The resulting reactive protein bands were visualized using a ChemiDoc™ MP Imaging System (Bio-Rad, USA).

Determination of specificity of anti-Ank mAb

To investigate whether Ank-94 mAb specifically recognizes the ankyrin structure, indirect ELISA was performed using a closely related scaffold, i.e., αRep 4E3 and 9A8.[21] Monomeric Ank1D4, αRep 4E3 and αRep 9A8 (1 µg/mL) were immobilized in a 96-well plate and incubated overnight at 4 °C in a moisture chamber. Wells were washed four times with PBST and blocked with 2% w/v BSA in PBS for 1 hr at RT. After being washed with PBST, proteins were incubated with the culture supernatant form of Ank-94 mAb (1:10 dilution) for 1 hr at RT. After being washed with PBST, a 1:3,000 dilution of goat anti-mouse Igs conjugated with HRP in 2% w/v BSA in PBS was added. The resulting specimen was subsequently incubated for 1 hr at RT. The binding activity was detected by adding a TMB substrate. The Degree of absorbance was then monitored at 450 nm.

Binding kinetics of anti-Ank mAb

The binding kinetics of Ank-54, Ank-59, and Ank-94 mAbs with monomeric and dimeric Ank1D4 were evaluated using bio-layer interferometry (BLI) with the BLItz™ system (FortéBio, Menlo Park, CA). Anti-Penta-HIS biosensors were pre-wetted for 15 min in buffer (2% w/v BSA in PBST) immediately before use. Recombinant H6-Ank1D4 (loading) at 5 µg/mL was immobilized to anti-Penta-HIS biosensors for 2 min. After being washed with buffer for 30 sec, the Ank1D4-loaded biosensor was dipped into a solution containing culture supernatant form of anti-Ank mAbs (association

step), followed by dipping in 2% BSA in PBST (dissociation step). The association (k_{on}) and dissociation rate (k_{off}) and the equilibrium dissociation constant (K_D) were calculated from a local fit to a 1:1 binding model of the data between anti-Ank mAbs and Ank1D4 using the BLItz Pro 1.1 software.

Purification of Ank-94 mAb using protein L affinity chromatography

Ank-94 mAb was seeded and expanded in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) under standard conditions (5% CO_2, at 37°C). The culture supernatant form of Ank-94 mAb was harvested and purified using affinity chromatography on the protein L matrix (GE Healthcare). The Ank-94 mAb supernatant was loaded onto a column and then washed with 20 ml of buffer (20 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.0). Neutralizing buffer (1 M Tris-HCl, pH 9.0) was prepared in collection tubes to be mixed with 1 mL of the eluted fractions. The antibody was eluted with 0.1 M glycine at a pH of 2.7, and fractions were selected at A280 nm. The eluted sample was eventually exchanged for the buffer to PBS.

Sandwich ELISA for ankyrin detection using Ank-94 mAb

96-well plates were immobilized with 1 µg/mL purified Ank-94 mAb and incubated at 4 °C overnight. After being washed with PBST, coated wells were incubated with 2% w/v BSA in PBS for 1 hr at RT. Recombinant monomeric or dimeric Ank1D4 (2 µg/mL) was added, followed by anti-His-HRP (1:3000 dilution, BioLegend) in 2% w/v BSA in PBS for 1 hr at RT. Ankyrin binding was detected by monitoring the ELISA plate reader at an absorbance of 450 nm after the TMB substrate was added.

Results

Isotyping of anti-Ank mAbs

All immunoglobulins were identified as IgG using IsoStrip. The Ank-54 and Ank-59 mAbs are IgG_1 kappa isotype, whereas Ank-94 mAb is IgG_{2a} kappa.

Binding activity of ankyrin antibody obtained from different hybridoma clones

Hybridoma culture supernatants containing anti-Ank mAb were performed to determine the binding activity against ankyrin immunogen. Indirect ELISA was then used to determine whether Ank-54, Ank-59, and Ank-94 mAbs could interact with the immunized Ank1D4 antigen. Among the three clones of anti-Ank mAb, the degree of binding activity of Ank-94 mAb was higher than for Ank-54 and Ank-59 mAbs (Figure 2).

Specific interactions of mAbs against ankyrin scaffold

The binding activity of mAbs was determined against irrelevant Ank2D3 and Ank1D4 scaffolds using indirect ELISA. Ank1D4 and Ank2D3 were targeted by Ank-54, Ank-59, and Ank-94 mAbs, which implied that the common antigenic determinant was specific (Figure 3).

Ank-94 mAb binding activity against dimeric Ank1D4

At mole equivalence of monomeric and dimeric Ank1D4, Ank-94 mAb demonstrated slightly higher signals in dimeric Ank1D4 (Figure 4). This result suggests that Ank-94 mAb could interact with both ankyrin forms.

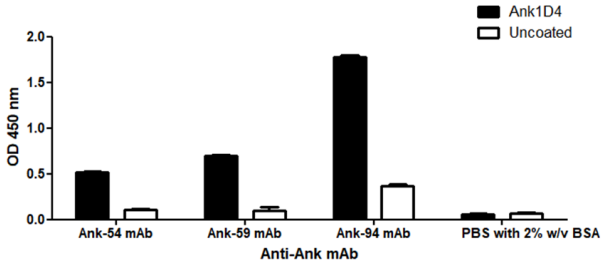

Figure 2. *Binding activity of mouse anti-Ank mAb. Different clones of mouse anti-Ank mAb interacted with immobilized Ank1D4. The binding activity of the three clones (Ank-54, Ank-59, and Ank-94 mAbs) of anti-Ank mAb and Ank1D4 antigen was determined with the use of HRP-conjugated goat anti-mouse Igs. Data were obtained from triplicate experiments and expressed as mean±SD values.*

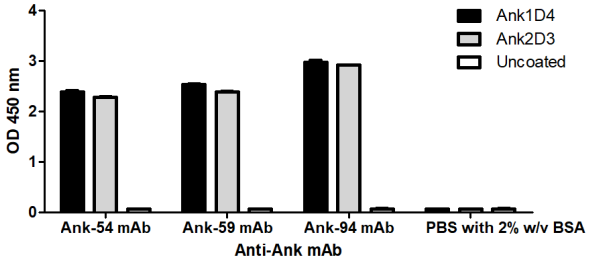

Figure 3. *An evaluation of binding characteristics of anti-Ank mAb. Ank-54, Ank-59, and Ank-94 mAbs was determined using indirect ELISA. Ank1D4 and Ank2D3 were immobilized in a 96-well plate following by anti-Ank mAb (Ank-54, Ank-59, and Ank-94 mAbs). Interactions with HRP-conjugated goat anti-mouse immunoglobulin were then detected. Data were obtained from triplicate experiments and expressed as mean±SD values.*

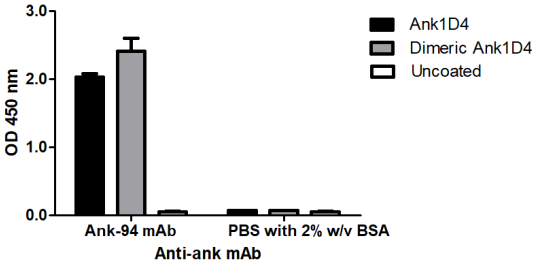

Figure 4. *Characterization of mouse anti-ankyrin monoclonal antibody clone 94. Ank-94 mAb against ankyrin was applied to detect monomeric and dimeric Ank1D4. Monomeric and dimeric Ank1D4 were immobilized on ELISA wells. After incubation, culture supernatant form of Ank-94 mAb were added and detected with HRP-conjugated goat anti-mouse immunoglobulin. Data were obtained from triplicate experiments and expressed as mean±SD values.*

Epitopic structure of ankyrins recognized by mAbs

Immunoblotting was performed to investigate whether anti-Ank mAb could interact with other ankyrin clones apart from Ank1D4. Ank2D3 was used for irrelevant proteins in lieu of Ank1D4. Ank-54, Ank-59, and Ank-94 mAbs demonstrated that the linearized recombinant Ank1D4 and Ank2D3 structures could be detected by all mAbs (Figure 5A). According to the amino acid alignment of Ank1D4 and Ank2D3 using Clustal Omega web server[22], conserved and variable regions of ankyrin scaffolds were depicted (Figure 5B). In addition, Ank-94 mAb recognized monomeric Ank1D4 (Figure 5C) and dimeric Ank1D4 (Figure 5D) in their denatured forms.

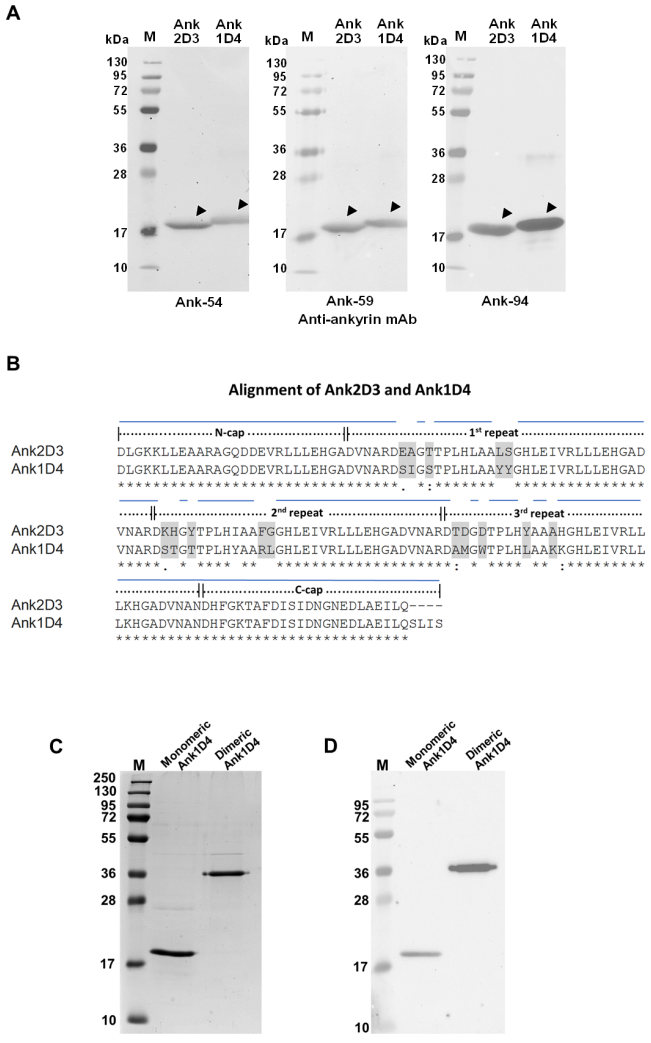

Figure 5. *Detection of anti-Ank mAb clones against ankyrin. (A) Ank-54, Ank-59, and Ank-94 mAbs detected both Ank2D3 and Ank1D4 using immunoblotting. (B) Schematic of alignment between Ank2D3 and Ank1D4. Amino acid sequences of Ank2D3 and Ank1D4 (Escherichia coli) were represented using the single letter code. Ankyrin repeats consisted of three repeat proteins flanked by the N- and C- terminus caps. Conserved and different variable residues (five positions) of ankyrins are represented in the blue line and highlighted in grey, respectively. Monomeric and dimeric Ank1D4 were separated using (C) SDS-PAGE and visualized by (D) immunoblotting using Ank-94 mAb as the primary antibody. Arrows indicate the major bands of ankyrin with M as a protein marker.*

Cross-reactivity testing of anti-ankyrin mAb with ankyrin and irrelevant protein

Indirect ELISA was performed to assess cross-reactivity between ankyrin and alpha-repeat proteins (αReps). When analyzing Ank-94 mAb, only the Ank1D4 protein was positive, while neither αRep 4E3 nor αRep 9A8 were detected in the anti-Ank mAb interaction (Figure 6). This finding suggests that anti-Ank mAb specifically interacted with ankyrin.

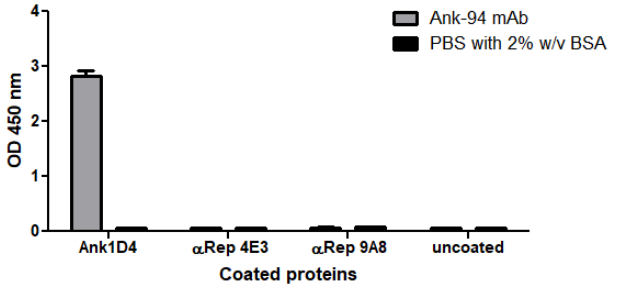

Figure 6. *Investigation of specificity of anti-Ank mAb. Cross-reactivity of Ank1D4 and two αReps was determined and detected by Ank-94 mAb using indirect ELISA. Ankyrin and αReps were immobilized in a 96-well plate followed by Ank-94 mAb. They were then detected with HRP-conjugated goat anti-mouse immunoglobulin. Data were obtained from triplicate experiments and expressed as mean±SD values.*

Binding kinetic properties of anti-Ank mAb clones against ankyrin

The binding kinetics of Ank-54 mAb, Ank-59 mAb, and Ank-94 mAb were displayed in the sensorgram (Figure 7). The equilibrium dissociation constant (KD) of the binding reaction between Ank-94 mAb (3.1×10^{-7} M) and Ank1D4 is stronger than Ank-54 mAb (4.8×10^{-8} M) and Ank-59 mAb (7.2×10^{-8} M).

Evaluation of binding preference of mAb against ankyrins

Of the three mAbs we prepared against ankyrin; Ank-94 mAb culture supernatant was purified via protein L affinity chromatography. Antibody sandwich ELISA was performed for comparisons between ankyrin monomer and dimer. The results indicated that immobilized Ank-94 mAb was able to capture monomeric and dimeric Ank1D4 following anti-His-HRP detection (Figure 8).

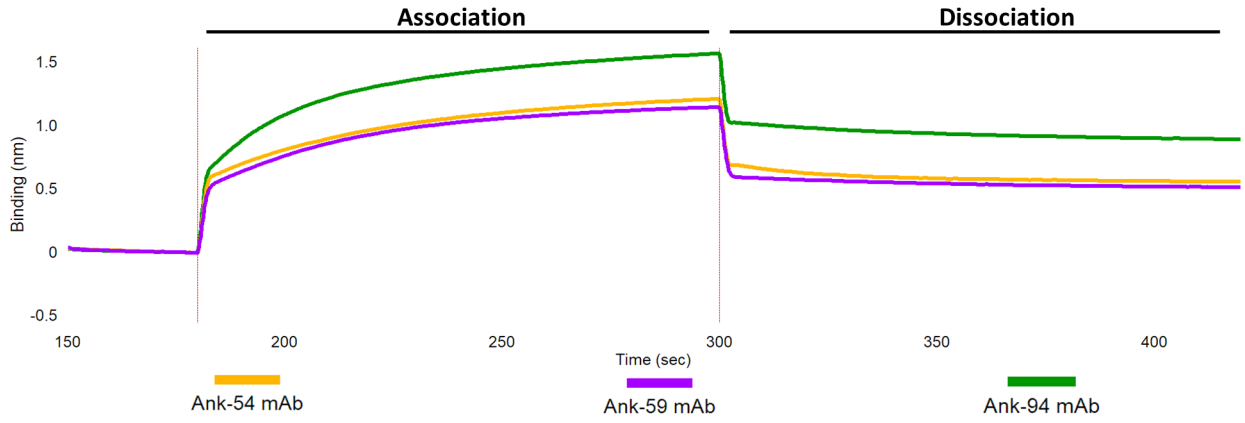

Figure 7. *The binding kinetics of anti-Ank mAbs displayed in the sensorgram. H_6-Ank1D4 was immobilized on anti-Penta-HIS biosensors and subsequently reacted with culture supernatant of Ank-54 (orange) or Ank-59 (purple) or Ank-94 (green) mAb.*

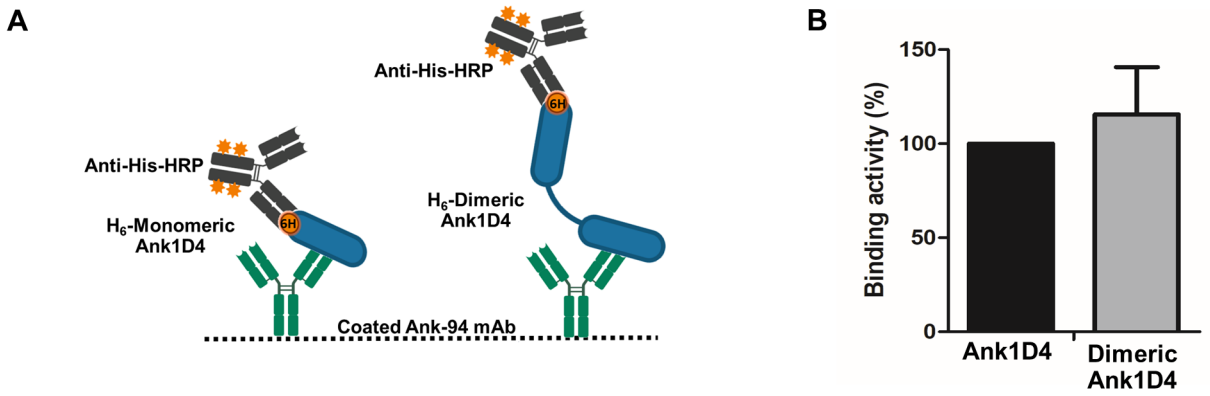

Figure 8. *Relative binding activity of purified Ank-94 mAb. (A) Schematic and (B) the percentage of antibody sandwich ELISA binding assay. Data were normalized with monomeric Ank1D4 levels to evaluate the binding activity using mathematical equation ($O_{Ddimer}/O_{Dmonomer}$)×100. This graph was produced from triplicate experiments. The results are expressed as mean±SD values.*

Discussion

The crucial role of scaffold proteins in cellular signaling pathways is required for accurate coordination.[23] Overexpression or deficiency of scaffolds can influence the development of certain diseases, such as the defects associated with tubulointerstitial fibrosis in renal failure.[3] Since a number of scaffold proteins are associated with pathogenesis, certain anti-scaffold antibodies have been established for immunological assays.[24–26] Among these, monoclonal antibodies specific to human ankyrins, i.e., ANK1, ANK2, and ANK3, have been developed.[8, 9, 27]

In our study, Ank-94 mAb was generated with the highest reactivity against the Ank1D4 immunogen. In addition, Ank-94 mAb demonstrated cross-specific reactivity with irrelevant Ank2D3 by indirect ELISA. This would suggest that anti-Ank mAb could interact with the conserved residues of DARPins in the pairwise alignment in Figure 4. The binding activity of Ank-94 mAb was further investigated for recognition with Ank1D4 dimer. The result indicates that Ank-94 mAb had reserved its binding function with Ank1D4 dimer. MAbs from three hybridoma clones, including Ank-94 mAb, were subsequently analyzed by immunoblotting. Their immunoreactivity demonstrated that the mAbs recognized the sequential epitope of either monomeric or dimeric Ank1D4 and Ank2D3. Notably, the alignment of Ank1D4 and Ank2D3 sequences indicate the presence of conserved residues of DARPins. Concerning the artificial structural proteins, conserved positions were defined for all the frameworks. Whereas non-conserved positions reflected an introduction of diversity.[16] Apart from specificity to the common epitope on DARPins, Ank-94 mAb did not exhibit non-specific activity with any other scaffold protein, i.e., αRep.

Apart from the specificity, Ank-94 mAb exhibited the highest binding affinity against Ank1D4. The binding kinetic of Ank-94 mAb (K_D=3.1 x 10^{-7} M) is stronger than Ank-54 and Ank-59 mAbs. It was therefore considered a good candidate for conferring to DARPins interaction. Generally, anti-Ank mAbs were generated to identify the natural ankyrins

(ankyrin-R, -B, and -G) expression that localized intracellular compartment for evaluation of ankyrin levels.[8,9,10] Antibodies selectively interacting with specific human ankyrins were generally established to directly evaluate ankyrin expression using immunoassays, i.e., immunoblotting or flow cytometry. Whereas Ank-94 mAb could be applied to trace protein-protein interactions to identify the specific target of DARPins in indirect ELISA. Ank-94 mAb recognized that the conserved residues occurred more than once in repeat modules. Thus, Ank-94 mAb provides an advantage of detection sensitivity compared to anti-His, which is generally used in tracing bound DARPins.[28] With regard to the consistency of mAb production and performance, Ank-94 mAb should be substituted for rabbit anti-ankyrin polyclonal antibodies to develop an immunochromatographic assay.[29] In addition, we established a sandwich ELISA to simulate the detection of DARPins. The result suggests that Ank-94 mAb captured His-tagged monomeric and dimeric Ank1D4 after being traced with anti-His-HRP. This system can be adapted to quantify other DARPins or native ankyrins that contain similar conserved residues.

Conclusion

Among the three clones of mouse monoclonal anti-ankyrin antibodies that could recognize DARPin, Ank-94 mAb demonstrated the highest degree of immunoreactivity. In addition to Ank1D4, Ank-54, Ank-59, and Ank-94 mAbs were bound with irrelevant Ank2D3 indicating that anti-Ank mAbs can interact with common residues. The binding characteristic of Ank-94 mAb relies on the linearized structure of ankyrins. No cross-reactivity with another scaffold, i.e., αRep, was observed. Notably, the specificity of Ank1D4 can assist future applications in discovering novel targets for candidate DARPins.

Conflicts of Interest

The authors declare no conflict of interest.

Ethical approval

Animal experiments were conducted following the animal use protocol approved by the Laboratory Animal Center, Office of Research Administration at Chiang Mai University. The Ethics Review Committee of the Laboratory Animal Center at Chiang Mai approved all animal experiments (Project number 2564/MC-002).

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