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Purification of Subdomains of the Plakin Repeat Domain in Microtubule Actin-crosslinking Factor 1b from Bacteria

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Abstract

MACF1 (microtubule actin-crosslinking factor 1; also known as ACF7) is a titanic cytoskeletal linker that can orchestrate different cytoskeleton elements to participate in various cellular processes. Our previous study (Lin et al, 2005) showed that MACF1b, a larger isoform of MACF1 that harbors additional plakin repeat domains, is involved in the maintenance of Golgi apparatus structure by targeting to Golgi apparatus. However, the underlying mechanisms of how MACF1b targets to Golgi apparatus and what interaction factors are involved in the function of MACF1b in Golgi apparatus remain to be determined. Here, we report the purification of two separate subdomains (MACF1b-PRD-N and MACF1b-PRD-C) of plakin repeat domains (PRDs) of MACF1b in order to further study the function of these PRDs of MACF1b in cells. Factors affecting the purification of these protein fragments are also discussed

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Introduction

MACF1 (microtubule actin-crosslinking factor 1), also known as actin-crosslinking family 7 (ACF7)[1], is a member of the spectraplakin family [2], which regulates dynamic cytoskeletal networks of actin microfilaments, microtubules and intermediate filaments. MACF1 is a large and conserved protein that comprises of multiple domains, which include the N-terminal actin-crosslinking calponin homology (CH) domain, membrane-targeting plakin domain, 23 flexible spectrin repeats, two calcium-binding EF hands, and C-terminal microtubule-binding domains (MTBD)[3, 4]. Alternatively spliced isoforms of MACF1 of somewhat different structures are also discovered[4, 5], including plakin-repeat domain-containing MACF1b [6]. These multiple domains of structure reflect their diversified biological functions in cells, which include cell-cell bridging, cell polarity, proliferation, cell migration, metastasis, epithelial-mesenchymal transition (EMT)[7], vesicular transport, osteoblast differentiation [8], and even signal transduction such as Wnt signaling [3, 9]. Recent evidences also indicate that MACF1 involves in a couple of pathological conditions such as neurodegenerative diseases [10], neuromuscular diseases [11], and cancers [12].

MACF1b (microtubule actin-crosslinking factor 1b) was originally discovered by homology search to look for more members of MACF1 (now MACF1a). MACF1b possesses additional plakin repeat domains that are located between the plakin domain and spectrin repeat domains of MACF1a. Molecular and biochemical evidences showed that MACF1b is ubiquitously expressed and implicated in maintenance of Golgi structure by targeting to Golgi apparatus [6]. Proteomics analysis of urine from cachectic gastro-esophageal cancer patients indicated that MACF1b could be a candidate biomarker of cancer cachexia [13]. Genomic analysis also showed that MACF1b is conserved across species [14, 15]. However, not too much progress was made for MACF1b since then. In this paper, we set out to express and purify two separate protein fragments of plakin repeat domain of MACF1b from bacteria for later functional analysis.

Materials and Methods

1. Materials

Yeast extract and tryptone were from Becton Dickinson (BD). Isopropyl β-D-1-thiogalactopyranoside (IPTG), glycine, and Tris base were from Amresco. Urea, glycerol, imidazole, bromophenol blue (BPB), Coomassie brilliant blue G-250, ammonium persulfate, dithiothreitol (DTT), and phenylmethanesulfonylfluoride (PMSF) were from Merck. 30% acrylamide and bis-acrylamide solution, Protein assay dye, and sodium dodecyl sulfate (SDS) were from Bio-Rad. N,N,N,N-tetramethyl-ethylenediamine (TEMED) was from Sigma. His Bind Resins were from Novagen.

2. Cloning of pET-MACF1b-PRD-N and pET-MACF1b-PRD-C

Mouse cDNAs encoding MACF1b-PRD-N (amino acids 1543-1966 of MACF1b, GenBank accession number: DQ067088) and MACF1b-PRD-C (amino acids 1961-2800 of MACF1b) subdomains were amplified by PCR from pKH3-FL-PRD [6], and cloned into *Nde*I and *Xho*I sites of pET-23a to make pET-MACF1b-PRD-N and pET-MACF1b-PRD-C respectively. The resulting proteins have a six in-frame histidines on the tails for protein purification. The clones were completely sequenced to make sure that there are no erroneous bases inside the clones.

3. Protein expression in bacteria

Empty vector, pET-MACF1b-PRD-C, pET-MACF1b-PRD-N were transformed into competent *E. coli* BL21 (DE3) bacterial strains by the heat shock method. Transformed colonies were inoculated into LB broth with antibiotics, shake and grew to reach OD_{600} of 0.6~0.8. IPTG, at a final concentration of 0.5 mM, was added into bacterial culture and shake for additional two hours. Bacteria were collected by centrifugation. Cell pellet was solubilized by Laemmli sample buffer, boiled for 5 minutes, spin at 12,000 rpm for 10 minutes to get the supernatant as the total proteins. An aliquot of proteins was separated by SDS-PAGE, and gels were stained with Coomassie brilliant blue G-250 to reveal the proteins.

4. Solubility test of expressed proteins

Transformed bacterial inoculum was seeded into 50 mL of LB/Ampicillin broth and grew to reach OD_{600} of 0.6~0.8. IPTG at a final concentration of 0.5 mM was added into bacterial culture for additional two hours for protein induction. Bacteria were then collected and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 0.1 mM PMSF), sonicated and centrifuged to obtain the supernatant as the soluble proteins. Cell pellet was further resuspended with Laemmli sample buffer, boiled and centrifuged to obtain the insoluble proteins. Both fractions were separated in SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250 to reveal the proteins.

5. Purification of His-tagged proteins from bacteria

Purification of MACF1b-PRD-C: As MACF1b-PRD-N and MACF1b-PRD-C harbor six in-frame histidine residues on the tails of protein fragments, nickel beads were used to purify these proteins. Transformed bacterial inoculum of pET-MACF1b-PRD-C was seeded into two liters of LB/Ampicillin broth and grew to reach OD_{600} of 0.6~0.8. IPTG at a final concentration of 0.5 mM was added into bacterial culture for additional two hours. Bacteria were then collected and resuspended in lysis buffer (0.5 M NaCl, 20 mM Tris-HCl, 6 M urea, 5 mM imidazole, and 0.1 mM PMSF), sonicated and centrifuged to obtain the supernatant as the crude lysate. Nickel resin

beads were washed thoroughly with sterilized water, and charged with 100 mM $NiSO_4$. The resins were equilibrated with binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 6 M urea, 5 mM imidazole, and 0.1 mM PMSF). Crude lysate was allowed to pass through the column for protein binding and the binding steps can be repeated for several times. The protein-bound resins were then washed extensively with wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 6 M urea, 40 mM imidazole, and 0.1 mM PMSF). The resins with bound proteins were then incubated in elution buffer (0.5 M NaCl, 20 mM Tris-HCl, 6 M urea, 150 mM imidazole, and 0.1 mM PMSF) for five minutes, and fractions of 0.5 ml were collected. Aliquots of purified proteins were mixed with Laemmli sample buffer, boiled and separated in SDS-PAGE, and gels were stained with Coomassie brilliant blue G-250 to reveal the proteins.

Purification of MACF1b-PRD-N: For the purification of MACF1b-PRD-N, bacterial pellet was resuspended in 8 M urea, sonicated, and incubated for overnight on a shaker at ambient temperature. After that, supernatant was collected by centrifugation and urea in supernatant was diluted to 1 M by urea-extraction buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 M urea, 5 mM imidazole, and 0.1 mM PMSF). All the steps after this step, including protein binding, wash, and elution, were the same as the purification of MACF1b-PRD-C protein fragments, except that 1M of urea was used in the buffer.

6. Dialysis and quantification of purified proteins

The purified MACF1b-PRD-C protein fragments were loaded into a dialysis bag and dialyzed against DTT-containing PBS with 4 M, 2 M, 1 M, and 0.5 M of urea sequentially, and finally dialyzed in sterile PBS with DTT. The purified MACF1b-PRD-N protein fragments were dialyzed against DTT-containing PBS with 0.5 M of urea first, and then dialyzed in sterile PBS with DTT. Dialyzed proteins were transferred to a new tube and glycerol was added into the purified proteins to a final concentration of 10%. Purified proteins were kept in -80^{o}C freezer for further analysis. An aliquot of purified protein fragments and a series of diluted bovine serum albumin (BSA) of known concentrations were boiled in Laemmli sample buffer, separated in SDS-PAGE, and proteins were revealed by staining in Coomassie brilliant blue G-250. The concentration of purified protein fragments were roughly estimated by comparing the density of purified proteins with the density of BSA.

Results and Discussion

1. Expression of MACF1b-PRD-C and MACF1b-PRD-N proteins in bacteria

To produce protein fragments of plakin repeat domain of MACF1b for functional

analyses, cDNAs corresponding to MACF1b-PRD-N and MACF1b-PRD-C subdomains were amplified by PCR from pKH3-FL-PRD [6], a mammalian cell expression plasmid with a full-length PRD cDNA. The resulting cDNAs with pre-engineered *Nde*I and *Xho*I sites were digested with restriction enzymes and cloned into the cognate sites of pET-23a to have pET-MACF1b-PRD-N and pET-MACF1b-PRD-C (Figure 1A) respectively. The protein fragments will harbor a six histidines on their tails to allow us to obtain the full-length expressed protein fragments after protein purification through nickel column. These expression vectors, together with empty vector, were then transformed into BL21 (DE3), an *E. coli* strain suitable for protein expression.

Transformed bacteria were cultured, induced by IPTG, collected and boiled in Laemmli sample buffer to have the total crude extract. Total proteins were subjected to SDS-PAGE, and the resulting gel was stained with Coomassie brilliant blue G-250 to reveal the fractionated proteins. The results showed that MACF1b-PRD-C protein fragments (indicated by an arrow head), an expected size of 92 kDa, and MACF1b-PRD-N protein fragments (indicated by an arrow), an expected size of 46 kDa, were expressed abundantly in transformed bacterial cells, as compared to those proteins from control bacterial cells with empty vector (Figure 1B). The MACF1b-PRD-C protein fragments display a much slower migration band obviously higher than the expected size of 92 kDa, which are very likely due to more amino acids of higher molecular weights exist in this protein fragment or protein modification (such as alkylation) of this fragment during boiling in Laemmli sample-buffer.

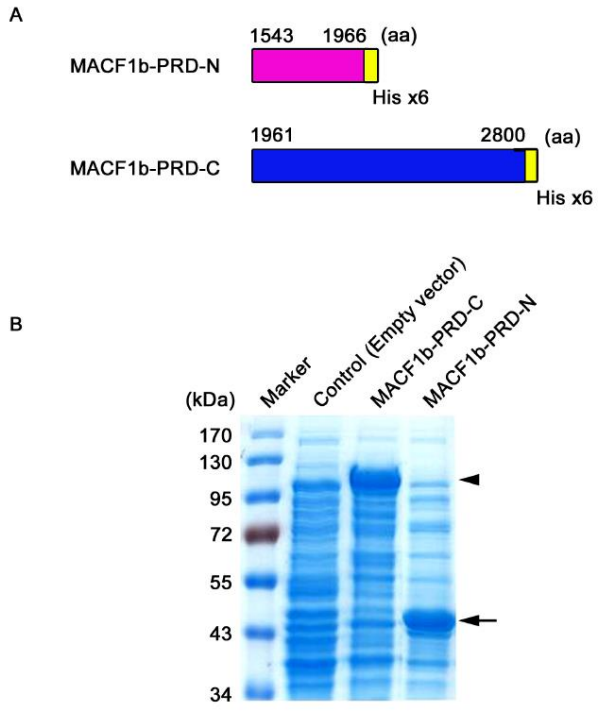

Figure 1: Expression of protein fragments of the plakin repeat domain in Microtubule Actin-crosslinking Factor 1b (MACF1b). (A) Schematic diagram of MACF1b-PRD-N (amino acids of 1543 to 1966 of MACF1b) and MACF1b-PRD-C (amino acids of 1961 to 2800) subdomains with six in-frame histidine residues. (B) Expression of MACF1b-PRD-N and MACF1b-PRD-C subdomains. Plasmids pET-MACF1b-PRD-N and pET-MACF1b-PRD-C were transformed into bacteria (*E. coli* BL21 (DE3)), and transformed bacteria were cultured and induced by IPTG (final 0.5 mM) for additional two hours and bacteria were collected by centrifugation. Cell pellets were solubilized in Laemmli sample buffer, boiled and centrifuged to obtain the soluble fraction. Proteins were fractionated by SDS-PAGE, and gels were stained with

Coomassie brilliant blue G-250 to reveal the proteins. The arrow head indicates the expressed MACF1b-PRD-C proteins, while the arrow indicates the expressed MACF1b-PRD-N proteins. Abundant proteins were induced by IPTG. Empty vector (pET-23a)-transformed bacteria were used as the control.

2. Solubility test of expressed proteins

As our target protein fragments were expressed abundantly in bacteria (Figure 1B), the next step is to determine whether these protein fragments were expressed in a soluble form, which will be much easier for isolation and purification. To this purpose, the transformed bacteria were cultured, induced by IPTG, collected, resuspended with lysis buffer, and sonicated. After centrifugation, the supernatant was collected as the soluble proteins. The resulting pellet was suspended in Laemmli sample buffer and boiled. The supernatant was collected after centrifugation as the insoluble proteins. The results showed that majority of both expressed protein fragments exist in the insoluble form (Figure 2, lanes 2 and 4). This data suggests that most of the expressed protein fragments would be very likely to go into the inclusion bodies, which structure consists of abundant aggregated insoluble proteins.

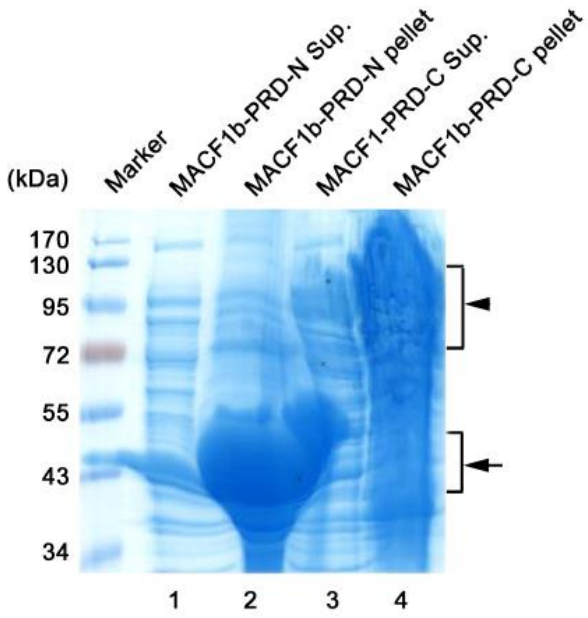

Figure 2: Solubility test of plakin repeats subdomains of Microtubule Actin-crosslinking Factor 1b (MACF1b). Plasmids pET-MACF1b-PRD-N and pET-MACF1b-PRD-C were transformed into bacteria (*E. coli* BL21(DE3)), and transformed bacteria were cultured and induced by IPTG (final 0.5 mM) for additional two hours and bacteria were collected by centrifugation. Cell pellet were solubilized with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 0.1 mM PMSF), sonicated and centrifuged to obtain the soluble proteins (labeled as supernatant; Sup.). The remaining pellet was furtherly extracted with Laemmli sample buffer by boiling and centrifuged to obtain the insoluble fraction (labeled as pellet). The gel was stained with Coomassie brilliant blue G-250 to reveal the proteins. The bracket with the arrow head indicates the expressed MACF1b-PRD-C proteins, while the bracket with the arrow indicates the expressed MACF1b-PRD-N proteins. Abundant expressed proteins were existed in the pellet fractions.

3. Purification of MACF1b-PRD-N and MACF1b-PRD-C proteins

In order to purify the protein fragments from inclusion bodies, urea-containing extraction buffer was used. For the purification of MACF1b-PRD-N protein fragments, transformed bacteria were cultured, induced by IPTG, collected and solubilized in extract buffer containing 8M urea. After repeated rounds of sonication, lysed

bacteria in urea extraction buffer were shaken overnight on a rotary shaker. Supernatant was collected after centrifugation, in which urea concentration was diluted to 1 M, and applied to nickel-charged column containing urea. After extensive washes, bound proteins were eluted with elution buffer containing 150 mM of imidazole. An aliquot of each fraction was separated by SDS-PAGE and the gel was stained with Coomassie brilliant blue G-250 to visualize the proteins. The results showed that the expressed protein fragments were extracted by urea (Supernatant; Lane 1 in Figure 3). Although some expressed protein fragments were in flow-through and wash fractions (Lanes 2 and 3 in Figure 3), abundant expressed MACF1b-PRD-N protein fragments were purified (Lanes 5 to 6 in Figure 3).

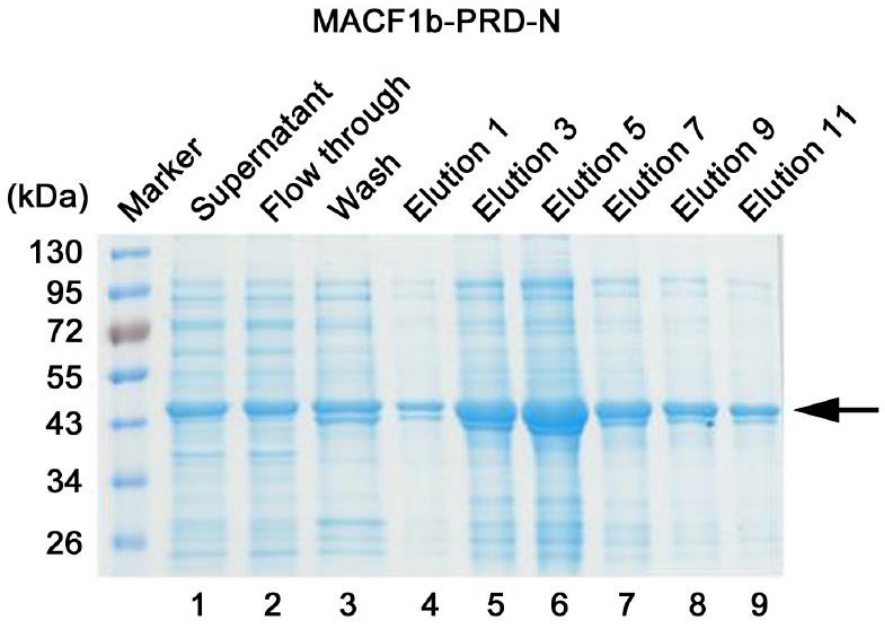

Figure 3: Purification of MACF1b-PRD-N protein fragments. Plasmid pET-MACF1b-PRD-N-transformed bacteria (*E. coli* BL21(DE3)) were cultured and used to inoculate two liters of LB broth. Bacteria were cultured, induced by IPTG (final 0.5 mM) for additional two hours and collected by centrifugation. Cell pellet was solubilized with urea extraction buffer (as shown in Methods), sonicated, and centrifuged to obtain the total proteins (supernatant). Total proteins were applied to the nickel-charged column and flow through was collected. The column was then washed and eluted with elution buffer containing 150 mM of imidazole. Aliquots of purified proteins were separated in SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250 to reveal the proteins. The arrow indicates the MACF1b-PRD-N protein fragments (expected size of 46-kDa).

For the purification of MACF1b-PRD-C protein fragments, extraction buffer containing 6 M of urea was used all the way from extraction, binding, wash, to elution. As shown in Figure 4, substantial amount of expressed proteins were solubilized by 6 M of urea (Lane 2 of Figure 4) after overnight incubation. Abundant MACF1b-PRD-C protein fragments were purified after extensive wash (Lane5 of

Figure 4). The elution kinetics of MACF1b-PRD-N (Figure 3) protein fragments were different from those of MACF1b-PRD-C protein fragments (Figure 4). The majority of purified MACF1b-PRD-C protein fragments were eluted at front fractions (Lane 5 of Figure 4), while most of MACF1b-PRD-N protein fragments were released at later fractions which peak around fraction 5 (Lane 6 of Figure 3).

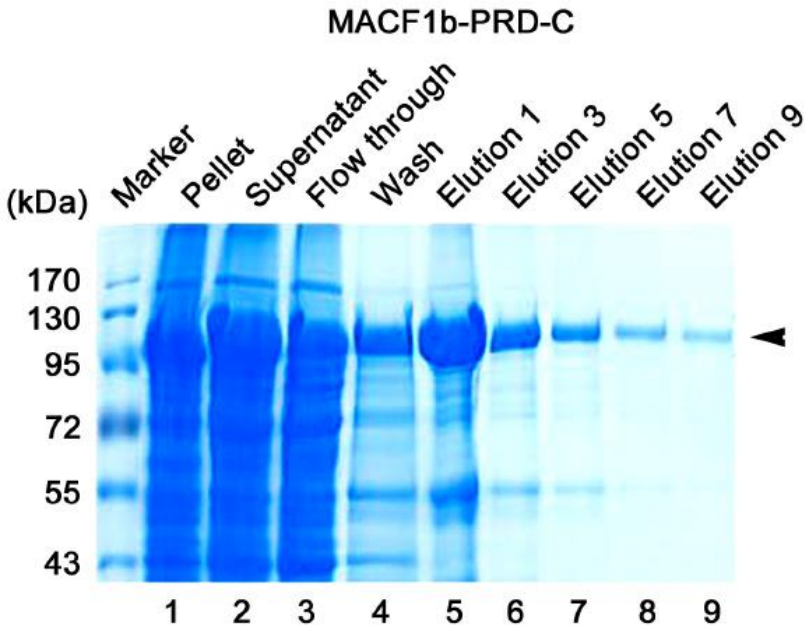

Figure 4: Purification of MACF1b-PRD-C protein fragments. Plasmid pET-MACF1b-PRD-C were transformed into bacteria (*E. coli* BL21(DE3)) and bacteria were cultured, induced by IPTG (final 0.5 mM) for additional two hours, and collected by centrifugation. Cell pellet was solubilized with urea extraction buffer (as shown in Methods), sonicated, and centrifuged to obtain the total proteins (supernatant). Proteins in the resulting cell pellet were solubilized by boiling in sample buffer (pellet). Total proteins (supernatant) were passed through the buffer-equilibrated nickel-charged column and flow through was collected (Flow through). Nickel-bound beads were then washed thoroughly with imidazole (40 mM)-containing wash buffer and the wash buffer was collected (Wash). Bound proteins were then eluted by elution buffer (0.5 M NaCl, 20 mM Tris-HCl, 6 M urea, and 150 mM imidazole) and fractions of 0.5 **ml were collected.** Aliquots of proteins were separated in SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250 to reveal the proteins. The arrow head indicates the MACF1b-PRD-C protein fragments, which mobility is a little bit different to the expected size (92-kDa).

4. Quantification of purified proteins

After re-folding of purified proteins by sequential dialysis, we set out to determine the approximate concentrations of the purified protein fragments. An aliquot of purified proteins, as well as a series of diluted bovine serum albumin (BSA) of known concentrations, were separated in the same SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250 to reveal the purified proteins. By comparing the density of purified protein with the density of a range of BSA standards, we estimated that the concentration of purified MACF1b-PRD-N protein

fragments to be around 0.65μg/μL (Figure 5), while that of MACF1b-PRD-C protein fragments to be about 0.33μg/μL (Figure 6). The purified protein fragments migrated as a major single band in the gel, suggesting that the purified protein fragments maintain good quality after sequential dialysis [9] and storage at -80°C.

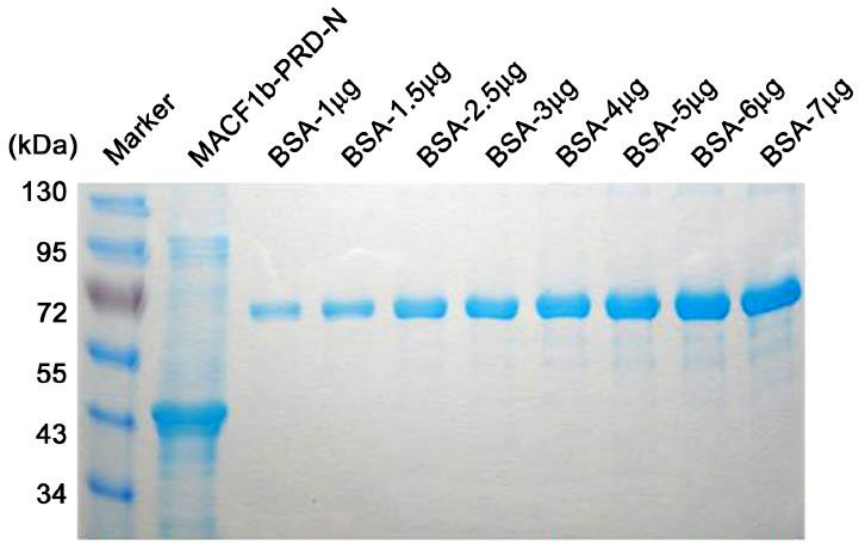

Figure 5: Quantification of MACF1b-PRD-N protein fragments. Purified MACF1b-PRD-N protein fragments, together with different concentrations of bovine serum albumin (BSA), were fractioned in SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250 to reveal the proteins. The concentration of purified MACF1b-PRD-N proteins were roughly estimated by comparing the density of purified proteins with the density of standard BSA proteins.

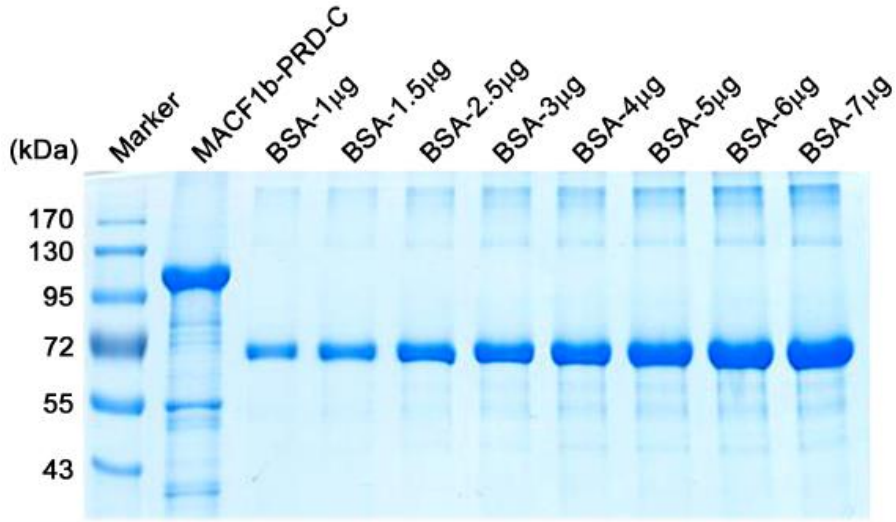

Figure 6: Quantification of MACF1b-PRD-C protein fragments. Purified MACF1b-PRD-C proteins fragments, together with different concentrations of bovine serum albumin (BSA), were fractioned in SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250 to reveal the proteins. The concentration of purified MACF1b-PRD-C proteins were roughly estimated by comparing the density of purified proteins with the density of standard BSA proteins.

In conclusion, we have successfully purified denatured MACF1b-PRD-N and MACF1b-PRD-C protein fragments and re-folding into a soluble form. Biochemical analysis and immunofluorescent staining showed that MACF1b specifically locates on the Golgi apparatus. The difference between MACF1b and MACF1a is the plakin-repeat domain (PRD), which is responsible for the Golgi-targeting activity of MACF1b [6]. Therefore, the PRD was picked for the current study. Given the important roles of Golgi apparatus in the modification of proteins and lipids, vesicle sorting, and targeting of cargos to its final destination including lysosome, plasma membrane, and extracellular space [16], it is crucial to know how MACF1b involves in these processes through interacting with some protein members of Golgi apparatus. We will use these two protein fragments for pull-down assays of lysates from enriched Golgi fraction to look for interaction proteins and how the interplay of MACF1b with these interaction partners for these biological processes will be further studied.

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