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Full Length Research Paper

Preparation and characterization of the polyclonal antibody against GAR domain of microtubule actin cross-linking factor 1 (MACF1)

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Growth arrest-specific 2 protein (Gas2) related domain (GAR domain), located at the C-terminal of microtubule actin cross-linking factor 1 (MACF1), plays a key role in microtubules (MTs) binding. To prepare the polyclonal antibody against GAR domain, cDNA encoding 466 amino acids protein of GAR domain was amplified from MG-63 cell by RT-PCR. The amplified cDNA, that exhibited 99% identity to the published sequence, was cloned into prokaryotic expression vector pQE-80L for the expression of GAR domain. Polyclonal antibody was then developed by inoculation of New Zealand rabbit with the recombinant GAR protein (rGAR) (97% purity). After several doses of immunization, the titer of antiserum reached 1: 62500 when evaluated by ELISA. The polyclonal antibody was further confirmed by Western blot, which gave results of high specificity and sensitivity (1: 5000). By using the polyclonal antibody to detect MACF1's association with MTs, laser scanning confocal microscopy (LSCM) showed that widely expressed MACF1 was partially aligned along MT filaments in the cytoplasm and co-located with MTs. This polyclonal antibody will be a valuable tool for the further studies of MACF1 and its GAR domain.

Key words: Microtubule actin cross-linking factor 1 (MACF1), GAR domain, clone, expression, purification, polyclonal antibody.

INTRODUCTION

Microtubule actin cross-linking factor 1(MACF1), which is a novel cytoskeletal linker protein and a member of spectraplakin family, possesses the structural characteristic of both plectin and dystrophin. It has an important role in the association with both actin microfilaments (AFs) and microtubules (MTs), and participates in signal transduction, protein transportation, embryonic development and disease occurrence (Chen et al., 2006; Sonnenberg and Liem, 2007; Leung et al., 2002; Jefferson et al., 2004). Recently, it uncovered an intrinsic actin-regulated ATPase domain in MACF1 and demonstrated that it is both functional and essential for these roles (Wu et al., 2008). A part of MACF1 cDNA was firstly isolated using a two-step degenerate PCR procedure and was named actin cross-linking family 7 (ACF7) by Byers et al. (1995). Subsequently, the full mouse ACF7 cDNA , alternatively named mouse actin cross-linking family 7 (mACF7) coding a 608 kDa protein was cloned (Bernier et al., 1996; Leung et al., 1999).

Two different formations of human MACF1 cDNA were also cloned and named trabeculin and macrophin, respectively (Okuda et al., 1999; Sun et al., 1999). Structurally, MACF1 contains three domains, namely an N-terminal domain binding to AFs, a rod domain and a C-terminal domain. At the COOH terminus, MACF1 contains two putative EF-hand calcium-binding motifs, as well as a region homologous to two related proteins, Gas2 and Gas2 related on chromosome 22 (GAR22) (Leung et al., 1999; Qian et al., 2008; Sun et al., 2000). Sun et al. (2000) found that the C- terminal of MACF1 contained at least two microtubule-binding regions; a GAR domain and a domain containing glycine-serine-arginine (GSR) repeats. The investigation also demonstrated that the C-

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terminal domain of MACF1 was bound to microtubules. The GAR domain was originally found in Gas2 protein and it could associate with microtubules in transfected cells. For the past few years, there have been increasing concerns about the effects of MACF1 on MTs. ACF7's cytoskeletal association was dependent on the microtubule network, but ACF7 also appeared to stabilize actin at sites where microtubules and microfilaments met (Karakesisoglou et al., 2000). Kodama et al. (2003) showed that ACF7 was an essential integrator of MT-actin dynamics and might work partly by adjusting the organization of the MT cytoskeleton to make connections with the actin cytoskeleton.

ACF7's actin and MT binding domains are required for normal MT trajectories and dynamics. Close attention was paid to MACF1 and its association to MTs. It was previously found that, under the simulated weightlessness condition produced by superconducting magnet with large gradient high magnetic field (LG-HMF), MACF1 was a sensitive protein related to skeleton; simulated weightlessness condition significantly influenced the expression and distribution of MACF1 (Qian et al., 2009a). MTs were fractured obviously but MACF1 still was co-located with relict MTs after the treatment by colchicine in MC3T3 and MG-63, which pointed out that the distribution of MACF1 in osteoblast relied on the integrity of the microfilaments (Qian et al., 2009b).

Given the results shown earlier, it has been confirmed that MACF1 is a significant protein related to cytoskeleton, especially the MTs. Preparation using synthetic N-terminal peptides of MACF1, globally commercial polyclonal antibodies against MACF1 were in very low quantities. Also, some products' reactivities and specificities were inadequate (former unpublished observations). Considering that there has not been any antibody against the C-terminal of MACF1 in the market so far, a polyclonal antibody was produced by cloning and expression of a confirmed domain of MACF1 as the antigen, and this was the GAR domain on the C-terminal of MACF1. In addition, this polyclonal antibody against GAR domain of MACF1 was further used in research on the biological structure and functions of MACF1 and its GAR domain.

MATERIALS AND METHODS

Amplification of GAR domain cDNA

According to the manufacturer's instructions, total RNA was extracted from MG-63 using TRIzol reagent (Invitrogen, USA) and the cDNA was synthesized using RNA PCR kit (TaKaRa, Dalian, China). Based on the cDNA sequence of the homo-MACF1 from the GenBank (ID: NM_ 012090), amplification for GAR domain cDNA was performed with specific primers, 5'-CGCGGATCC CAGACATTTATGGAGGAGATG-3' and 5'-CCCAAGCTTTAATC GCTTGGGACCTGGAG-3' (Sangon, China) and *Ex Tag* DNA polymerase (TaKaRa, Dalian, China) to minimize mutations. The PCR products were gel purified (Qiagen, Germany) and then cloned into pMD 18-T (TaKaRa, Dalian, China) vector by T-A clone. Firstly, they were examined by PCR using specific primers and by double digestion using *Bam*H I and *Hin*d III (TaKaRa, Dalian, China), and the positive recombinants were sent for sequencing.

Expression of rGAR

GAR domain cDNA was subcloned into pQE-80L vector (Qiagen, Germany) which contained 6 consecutive histidine residues (6×His tag) for the purification of 6×His-tagged proteins by affinity chromatograph. The positive bacterial colony was cultured in 10 ml LB/Amp$^+$ medium at 37℃ overnight, and then subcultured in fresh LB/Amp$^+$ medium with the ratio of 1:100. When the optical A_{600} reached 0.6 ~ 0.8, IPTG (Amresco, USA) was added into the medium to make a final concentration of 1 mmol/l. The bacteria were further cultured at 37℃ for about 4 h. SDS-PAGE and Western blotting were performed for the analysis of the expression of rGAR.

Purification of rGAR

2 L *Escherichia coli* DH5α (Tiangen, China) were induced. They were collected by centrifugation at 6500 r/min for 10 min. The collected bacteria were washed with PBS and lysed by sonication in the lysis buffer containing 50 mmol/l Tris-HCl (pH 8.5), 100 mmol/l NaCl, 0.5% Triton X-100 and 0.1 mmol/l PMSF. After centrifuging the lysates at 10000 r/min for 10 min, the collected pellets were homogenized on ice-bath condition in the wash buffer I containing 50 mmol/l Tris-HCl (pH 8.5), 50 mmol/l NaCl, 0.5% Triton X-100, 5 mmol/l DTT and 2 mol/l urea. The suspension was centrifuged at 10000 r/min for 10 min after standing for 30 min. The supernatant fluid was discarded and this step was repeated for another 2 times using wash buffer I and wash buffer II (50 mmol/l Tris-HCl (pH 8.5), 50 mmol/l NaCl, 0.5% Triton X-100, 5 mmol/l DTT and 4 mol/l urea), respectively. At last, the pellets were denatured in the dissolution buffer containing 20 mmol/l sodium phosphate, 500 mmol/l NaCl, 8 mol/L urea, pH 7.4 at 4℃ overnight. rGAR were firstly purified by HisTrap HP (GE, USA) referring to the instruction and then by gel purification. The later procedure was by separating the rGAR with SDS-PAGE and staining the protein bands by pre-cooled 0.2 mol/l KCl. The gels that contained rGAR were cut off, ground and soaked in physiological saline overnight. The supernatant that contained rGAR were collected by centrifugation, and then the purity was analysed by SDS-PAGE.

Preparation of polyclonal antibody against rGAR

New Zealand rabbit was used to produce the polyclonal antibody. For initial injection, 0.8 mg rGAR in a volume of 0.8 ml was mixed and emulsified with equal volume of complete Freund's adjuvant (Sigma, USA). The mixture was injected subcutaneously at multiple sites on the back of the rabbit. Three boost injections were performed on the 21th, 35th and 49th day after the initial injection. For every boost injection, 0.5 mg purified rGAR was used along with the incomplete Freund's adjuvant (Sigma, USA). 14 days after the forth injection, the rabbit antiserum was collected.

Sensitivity analysis of antiserum by ELISA

The antiserum titer was monitored by ELISA and serum was separated before immunity was used as the negative control. Sera were diluted in PBS (containing 1% BSA) by 500, 2500,12500, 62500, 125000 and 250000-fold, respectively, and was reacted with the purified rGAR which was used as the coating antigen. Goat anti rabbit IgG-HRP (Beijing Biosynthesis Biotechnology, China) and TMD were used as the secondary antibody and the chromogenic reagent. A450 nm values were read by Synergy HT Multi-MODE

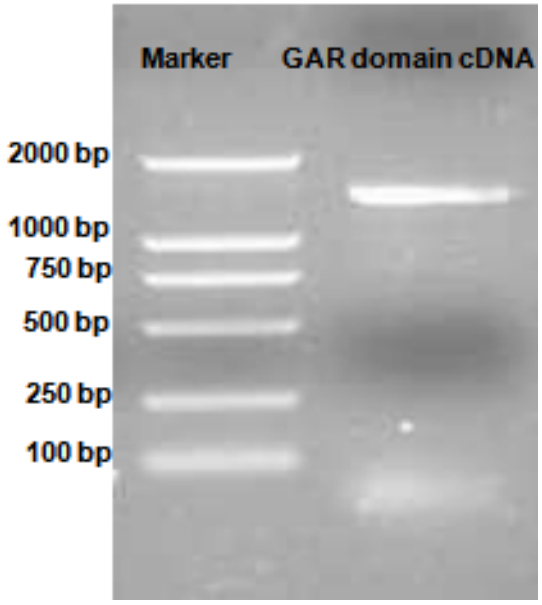

Figure 1. Amplification of GAR domain cDNA. The cDNA encoding GAR domain was amplified using RT-PCR and detected by 1% agarose electrophoresis; 1416 bp of GAR domain cDNA was conspicuously observed and the size was consistent with the expectation.

microplate reader (BioTek, USA).

Purification of the polyclonal antibody against rGAR

The antiserum was precipitated by 33 and 50% saturation ammonium sulfate sequentially, and filtered by 0.22 μm filter membrane. Ion-exchange chromatography was performed by HiTrap DEAE FF (GE, USA), with start buffer (Tris-Hcl, pH 8.3) for first washing step and elution buffer (Tris-Hcl, 1 mol/L Nacl, pH 8.3) for the second collection step. Finally, the collected antibody was dialyzed against PBS.

Specificity analysis of polyclonal antibody by Western blot

SDS-PAGE of the induced and non-induced DH5α lysates were performed and the whole bacterial proteins were transferred to NC membrane. Polyclonal antibody against rGAR (1:5000) and IRDye800 conjugated anti-rabbit IgG (1:4000) (Rockland, USA) were used as the first and secondary antibodies. The NC membrane was scanned simultaneously at 700 and 800 nm by Odyssey Infrared Imaging System (700 nm detection for normalization stains, and 800 nm detection for IRDye800 conjugated anti-rabbit IgG) (LI-COR, USA).

Specificity analysis of the polyclonal antibody by laser scanning confocal microscopy

After been cultured on the sterile cover glasses for 12 h, MG-63 cells (1×10^5/ml) were fixed with methanol (-20°C), washed with PBS, blocked with Abdil reagent (PBS containing 2% BSA, 0.1% Triton X-100 and 0.1% Azide) and incubated with polyclonal antibody against rGAR (1:20) and anti-α-tubulin mouse mAb (1:20) (EMD Chemicals, Germany) for 1 h at room temperature. They were washed with PBS-TX (PBS containing 0.1% TritonX-100) and MG-63 cells were incubated with rhodamine-labeled anti-mouse IgG (1:10) and FITC-labeled anti-rabbit (1:10) (KPL, USA). Then, the cells were enveloped with glycerol (Beyotime, China) and examined using Leica TCS SP5 laser scanning confocal microscope (Leica, Germany). FITC (green fluorescence) was excited at a wavelength of 488 nm and rhodamine (red fluorescence) at 543 nm.

RESULTS

Amplification of GAR domain cDNA

The cDNA encoding GAR domain was amplified using RT-PCR and detected by 1% agarose electrophoresis. 1416 bp of GAR domain cDNA was conspicuously observed and the size was consistent with the expectation (Figure 1). The sequencing result showed that GAR domain cDNA had 99% identity to the published sequence and no amino acid changed.

Expression and purification of rGAR

The DH5α containing pQE80L-GAR was induced by IPTG, lysed and analysed by SDS-PAGE and Western blot. In comparison with the non-induced DH5α, a distinct protein band between M_r 45000 and 66200 appeared in the induced DH5α and it matched the theoretical molecular weight M_r 52000 of rGAR (Figure 2A). SDS-PAGE also showed that rGAR almost existed in the precipitate of the lysed bacteria, which suggested that the expressed rGAR was almost the inclusion body (Figure 2B). Using anti-his_6 antibody, Western blot also showed the specific band at the estimated rGAR molecular weight in the induced DH5α, but not in non-induced DH5α and induced DH5α containing pQE-80L (Figure 2C). 97% purity rGAR was obtained after affinity chromatograph and gel purification, while 65% purity was obtained after only affinity chromatograph (Figure 2D).

Purification of polyclonal antibody against rGAR

Figure 3 shows the results of the SDS-PAGE for determining the purity of IgG. Two distinct protein bands with molecular weight of about 55 and 25 kDa corresponded to the rabbit IgG heavy chain and light chain. With purification by ion-exchange chromatography (HiTrap DEAE FF), 97% purity rabbit IgG was obtained.

Sensitivity analysis of antiserum by ELISA and specificity analysis of polyclonal antibody by Western blot

ELISA was carried out using antiserum collected after 4 times inoculation and serum was collected before

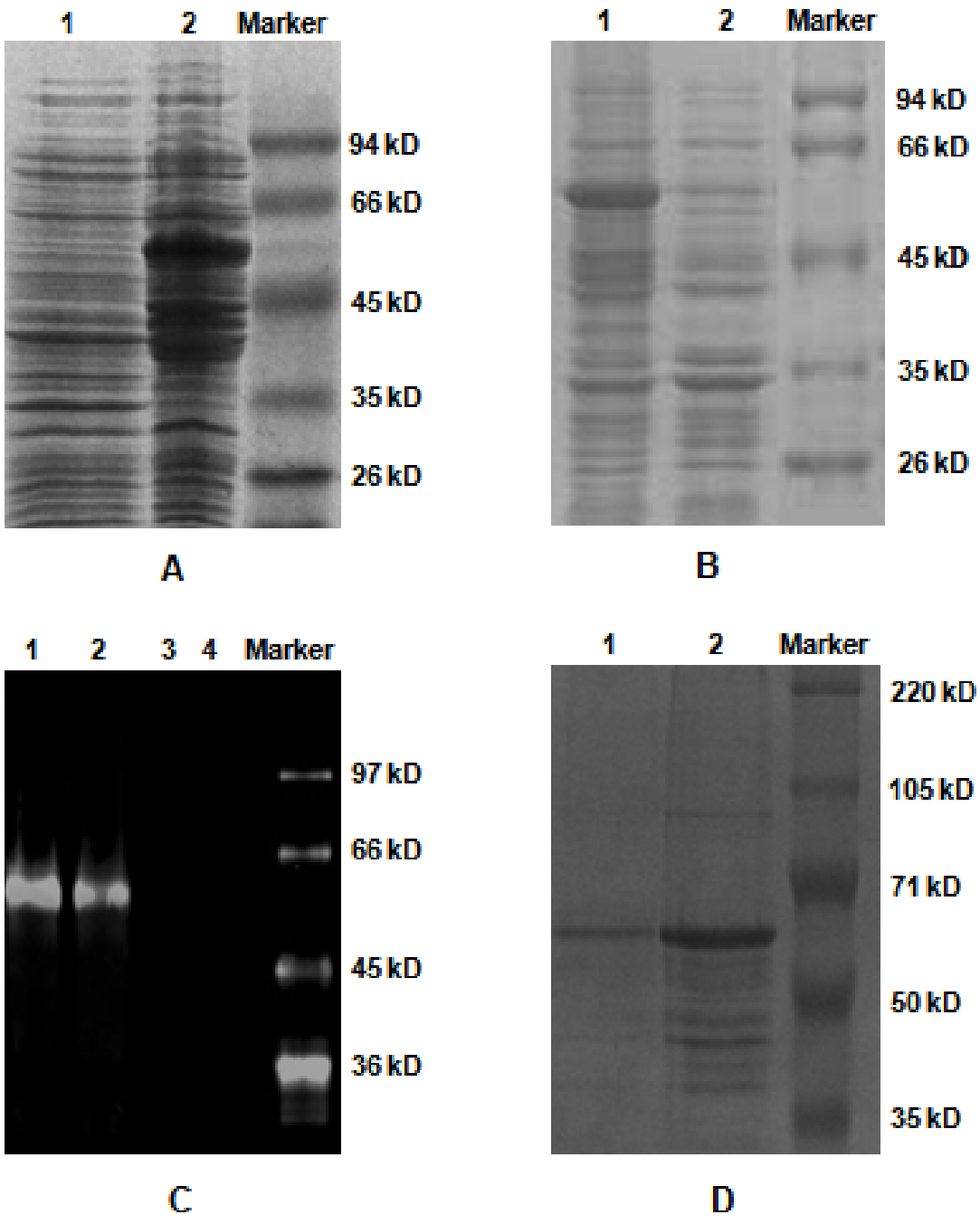

Figure 2. Expression and purification of rGAR. Distinct 52 kD rGAR was expressed by inducement of 1 mmol/l IPTG (A, lane 2), but was not expressed without inducement (A, lane 1). B shows that the precipitate of lysate (lane 1) contained much more rGAR when compared with the supernatant of lysate (lane 2), which suggested that the expressed rGAR was almost the inclusion body. In Western blot, anti-His_6 antibody detected rGAR in the induced DH5α (C, lanes 1 and 2) but not in the non-induced DH5α (2C, lane 3) and induced DH5α containing pQE-80L (C, lane 4). 65% purity rGAR was obtained after affinity chromatograph (D, lane 2) and 97% purity was obtained after affinity chromatograph and gel purification (D, lane 1).

inoculation as the control. Positive criterion was defined as $A_{antiserum}/ A_{control} \geq 2.1$. ELISA showed that high titer antiserum (1:62500) was acquired after 4 times inoculations (Figure 4). Furthermore, the specificity of polyclonal antibody was analyzed by Western blot; after dilution to 1:5000, the purified antibody could still recognize the specific rGAR expressed in the induced DH5α, but could not recognize any protein in non- the induced DH5α.

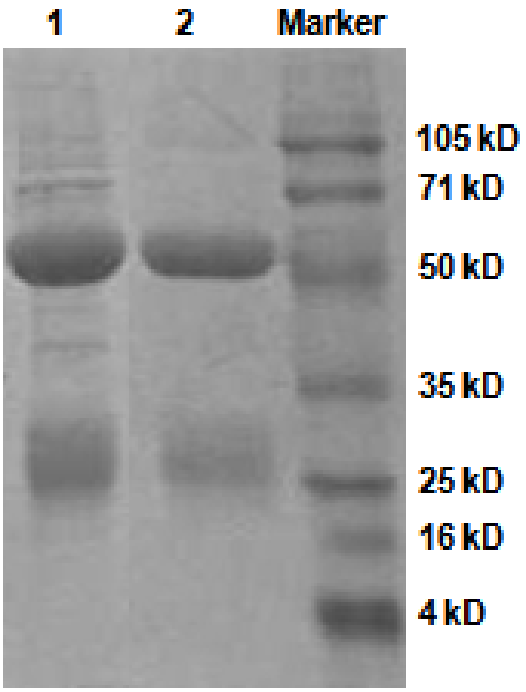

Figure 3. Purification of polyclonal antibody against rGAR. Lane 1 shows the purification result after precipitation by 33 and 50% saturation ammonium sulfate. With ammonium sulfate and ion-exchange chromatography (HiTrap DEAE FF), 97% purity IgG was obtained. The 55 and 25 kDa bands represent IgG heavy chain and light chain (Lane 2).

(Figure 5).

Specificity analysis of polyclonal antibody by laser scanning confocal microscopy (LSCM)

By using the polyclonal antibody to detect MACF1's association with MTs, LSCM obviously showed that, MACF1 was widely expressed in MG-63; MACF1 was punctate or flocculent in shape and mainly distributed in cytoplasm of MG-63 (Figure 6B); MACF1 (green) partially aligned along microtubules (red) and co-located with microtubules in MG-63; and the colocalizations were yellow showed (Figure 6C).

DISCUSSION

The GAR domain cDNA was amplified and sequenced. The sequence analysis indicated that the nucleic acid accuracy was as high as 99%, with no amino acid sequence change. These made the expressed GAR proteins to be available for the next steps. pQE-80L expression vector was chosen in this study: it was a high yield vector and the output of target proteins reached up to 50% of bacterial total proteins. Besides, pQE-80L contained 6xHis tag which facilitated the affinity purification of the target proteins (Zhang et al., 2006). In this study, the expression of rGAR reached 31% of the total proteins and rGAR was the highest expression protein in the induced DH5α. 84% rGAR was expressed in the form of inclusion body in this work. It is generally accepted that inclusion body formed because the new expressive proteins were not able to fold accurately without enough factors, which existed in the host cells and helped proteins to fold; in order to protect themselves from poisoning or death, the host cells chose to express the exotic proteins in the form of inclusion body since quantities of target proteins were probable toxic or lethal. The form of inclusion body still had some advantages, such as preventing target proteins from been degraded by intra-cellular proteases, bringing convenience to cell lysis procedure because of their insensitive to mechanical agitation and ultrasonication, getting higher expression level and higher purity.

While 49% purity of rGAR was obtained without the wash steps, 65% purity was obtained with the wash steps before affinity purification. Obviously, they did not meet the need for immunizing animals. Subsequent gel purification was proved available since high titer of antiserum and high specificity of purified polyclonal antibody were quantified by ELISA (1:62500) and identified by Western bolt (1:5000). The results demonstrated that polyclonal antibody against the GAR domain of MACF1 were prepared successfully.

Specificity of polyclonal abtibody identified by LSCM showed that, the widely expressed MACF1 was partially aligned along microtubule and was co-located with microtubules in the cytoplasm. This was in accordance with the research conclusions of Bernier et al. (2000) and Karakesisoglou et al. (2000). MACF1 was the first protein verified to be widely expressed and that could bind microfilament with microtubule cytoskeleton (Bernier et al., 2000). MACF1 had at least one actin and one microtubule binding domain and was partially co-located with microfilament and microtubule cytoskeleton; ACF7 discontinuously decorates the cytoskeleton at the cell periphery, including microtubules and actin filaments (Karakesisoglou et al., 2000). All these made the polyclonal antibody an adequate polyclonal antibody for further investigation of MACF1 and GAR domain.

In summary, GAR domain cDNA and rGAR were successfully amplified and expressed. Additionally, the high sensitive and specific polyclonal antibody was developed successfully. This adequate polyclonal antibody will be a useful tool for the studies on MACF1 and its GAR domain in the future.

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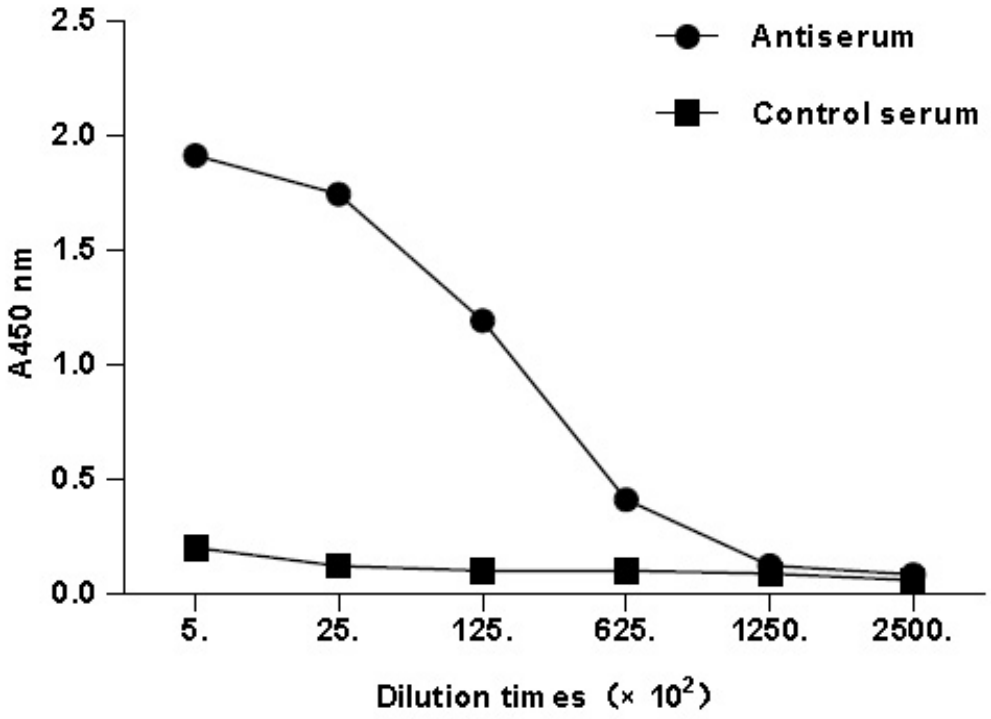

Figure 4. Sensitivity analysis of antiserum by ELISA. ELISA was carried out using antiserum collected after 4 times inoculation and serum before inoculation as the control. Positive criterion was defined as $A_{antiserum}/ A_{control} \geq 2.1$. The result showed that the high titer antiserum (1:62500) was acquired after 4 times inoculations.

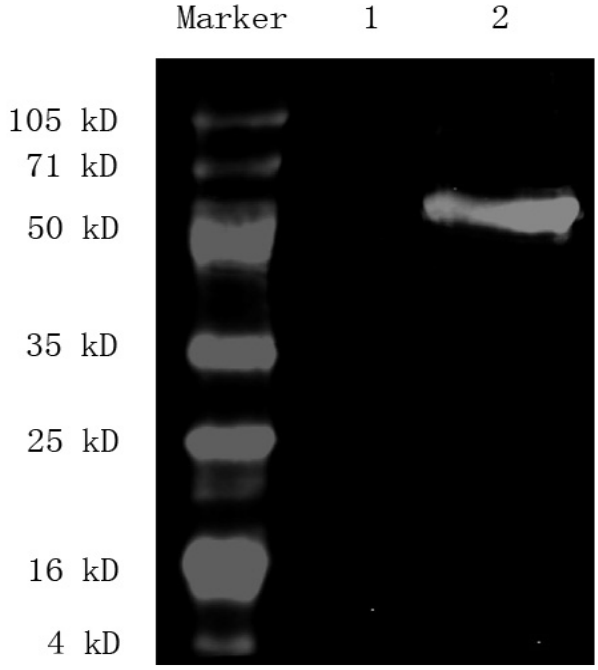

Figure 5. Specificity analysis of polyclonal antibody by Western blot. After diluted to 1: 5000, the 97% purified antibody could still recognize the specific rGAR expressed in induced DH5α (Lane 2), but could not recognize any protein in the non-induced DH5α (Lane 1).

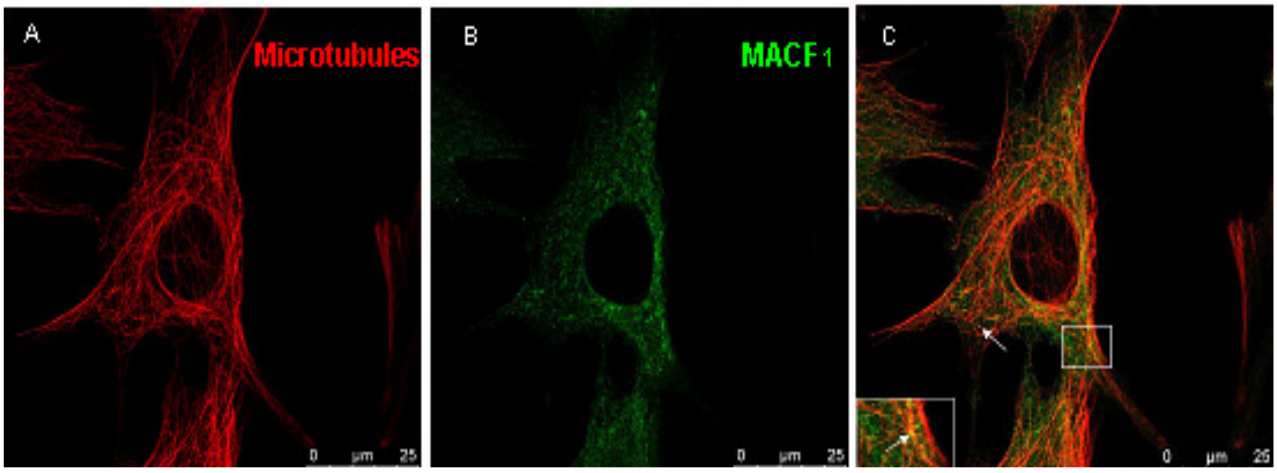

Figure 6. Specificity analysis of polyclonal antibody by laser scanning confocal microscopy (LSCM). A, shows the microtubules (red) of MG-63. By using the polyclonal antibody to detect MACF1's association with MTs, LSCM obviously indicated that the widely expressed MACF1 (green) was punctate or flocculent in shape and was mainly distributed in the cytoplasm of MG-63; (B), MACF1 partially aligned along microtubules and co-located with microtubules in the cytoplasm. The colocalizations were yellow; (C), Arrowhead in C denoted the colocalization of MACF1 and MTs. Inset , higher magnification to illustrate the colocalization more clearly.

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