



Development and Characterization of a Specific Anti-Caveolin-1 Antibody for Caveolin-1 Functional Study in Human, Goat and Mouse

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ABSTRACT : Caveolin-1 of the caveolin family of proteins regulates mammary gland development and has been shown to play a contradictory role in breast tumor progression. A specific anti-Caveolin-1 antibody will be useful for functional study of Caveolin-1 in different tissues. In this study, we generated a rabbit polyclonal antibody that specifically recognizes the N-terminal amino acids 50-65 of Caveolin-1. This polyclonal antibody specifically reacted with Caveolin-1 extracted from cells of different species, including human epithelial A431 cells, goat primary mammary epithelial cells and mice fibroblast NIH 3T3 cells, by Western blotting. Endogenous Caveolin-1 protein expressing in cells and normal human tissues was detected by this polyclonal antibody using immunocytofluorescent and immunohistochemical staining, respectively. Furthermore, an apparent decrease in Caveolin-1 expression in tumorous breast and colon tissues was detected by this polyclonal antibody. In conclusion, we have identified amino acids 50-65 of Caveolin-1, which contains an epitope that is specific to Caveolin-1 and is conserved in the human, goat and mouse. In future, this anti-Caveolin-1 antibody can be used to examine the progression of breast and colon cancers and to study functions of Caveolin-1 in human, goat and mouse cells. (**Key Words :** Caveolin-1, Peptide Antibody, Breast Cancer, Goat, Cancer Marker)

INTRODUCTION

Caveolin-1, a 21-24 kDa vesicular membrane integral protein containing 178 amino acid residues, is enriched in caveolae where invaginated membrane microdomains accumulate cholesterol, glycosphingolipids and receptors for many signal molecules (Rothberg et al., 1992; Okamoto et al., 1998). Both the N- and C- terminal of Caveolin-1 orient toward the cytoplasm and hydrophiles, while the hydrophobic central stretch of the protein is embedded in membrane (Krajewska and Maslowska, 2004). The C-terminal domain (residues 135-178) is essential for protein-protein interaction and membrane attachment. Caveolins' N-terminal region contains the Caveolin scaffolding domain (CSD; residues 82-101) which is necessary for its interaction with signal transducing molecules related to cell growth (Sargiacomo et al., 1995; Okamoto et al., 1998) including G-protein (Li et al., 1996), Ha-Ras (Song et al.,

1996a), Src family tyrosine kinases (Li et al., 1996), eNOS (Ju et al., 1997), EGFR (Couet et al., 1997), receptor tyrosine kinases (Engelman et al., 1997; Wary et al., 1998) and serine/threonine kinases (Razani and Lisanti, 2001). Caveolin-1 has been suggested to act as a negative regulator of surface membrane signaling molecules through its interaction with Caveolin scaffolding domain (Krajewska and Maslowska, 2004).

Caveolin-1 expression is dramatically down-regulated in mammary gland during late pregnancy and lactation through prolactin signaling cascade in mouse (Park et al., 2001). Interestingly, overexpression of recombinant Caveolin-1 in mammary epithelial cell line HC11 inhibits prolactin-induced activation of β -casein promoter activity and its synthesis (Park et al., 2001). Caveolin-1 null mice show acceleration of premature development of the lobulo-alveolar compartment of mammary gland during pregnancy and delay mammary gland involution (Park et al., 2002; Li et al., 2006). These results indicate Caveolin-1 negatively regulate mammary development and lactation.

The role of Caveolin-1 in suppressing tumorigenesis has emerged from the successful cloning of Caveolin-1: using mRNA subtractive hybridization and differential display to screen genes highly expressed in normal human mammary

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epithelial cells but lower in mammary adenocarcinoma-derived cells (Sager et al., 1994). Caveolin-1 expression is lost or down-regulated in both transformed human epithelial cell lines (Koleske et al., 1995) and ectopic oncogene-transformed cells (Engelman et al., 1997; Engelman et al., 1998) compared to normal cells. Ectopic expression of recombinant Caveolin-1 in metastatic mammary adenocarcinoma cells (MTLn3) blocked their anchorage-independent growth in soft agar, reducing their metastatic potential (Zhang et al., 1998). Absence of Caveolin-1 expression in mouse breast tissue was more susceptible to carcinogen-induced tumorigenesis (Park et al., 2002; Capozza et al., 2003), indicating its importance for tumorigenesis. These experiments provided circumstantial evidence that Caveolin-1 possesses anti-tumorigenic activity.

Using loss of heterozygosity (LOH) analysis and a specific polymorphic CA repeat microsatellite marker (D7S522), Caveolin-1 was proved to locate on human chromosome 7q31.1 (Engelman et al., 1999); a commonly deleted region in many kinds of human epithelial cancers including human primary breast (Zenklusen et al., 1994a), prostate (Zenklusen et al., 1994b; Jenkins et al., 1998), ovarian (Kerr et al., 1996), colon and renal cell carcinomas (Shridhar et al., 1997). Deletion mutation of Caveolin-1 in human chromosomes suggests a link between loss of Caveolin-1 expression and tumorigenesis.

Many newly discovered paragraphic GC islands are distributed on the Caveolin-1 promoter, and the first and second intron regions (Lee et al., 2002). Methylation of the GC islands in the promoter region of Caveolin-1 resulted in loss of Caveolin-1 expression in human breast cancer cell lines (MCF7 and T-47D) (Engelman et al., 1999), small cell lung cancer (Sunaga et al., 2004), breast cancer (Chen et al., 2004), prostate cancer (Cui et al., 2001), sporadic colorectal cancer (Lin et al., 2004) and ovarian carcinoma (Wiechen et al., 2001). These data suggests down regulation of activity of Caveolin-1 promoter with progression of tumorigenesis.

However, a growing body of evidence suggests that Caveolin-1 is up-regulated in primary prostate cancer and metastatic prostate carcinomas, esophageal squamous cell carcinoma, mammary adenocarcinoma, lung adenocarcinoma and clear cell renal cell carcinomas (Yang et al., 1998; Nestl et al., 2001; Ho et al., 2002; Kato et al., 2002; Joo et al., 2004). On the other hand, increase of Caveolin-1 expression is associated with cancer cells which acquisition of multidrug resistance (MDR) (Lavie et al., 1998). Together, all these finding implicate that Caveolin-1 has a "biphasic" expression pattern and function for different adaptive cellular response in tumorigenesis. Therefore, Caveolin-1 has the potential to act as a useful marker for tumor progression.

Breast and colon cancers in human were reported to

have decreased expression of Caveolin-1 particularly found in the Oriental world (Chen et al., 2004; Lin et al., 2004). Current methods for diagnosing breast cancer included mammography, flow cytometry and immunoperoxidase techniques, which can detect positive expression of estrogen receptor, progesterone receptor, C-erb B2 and Cathepsin D in breast cells (Barnes et al., 1996; Seol et al., 2006). Unfortunately, no single method developed thus far can yet detect the progression tumorigenesis over time. Usually it is necessary to combine several methods for more accuracy.

The objectives of this study were to develop a Caveolin-1 polyclonal peptide antibody for functional study of its role in mammary gland of many species as well as to detect its expression level in tumorous breast tissues. The endpoint of this study is to utilize the antibody for investigation of the Caveolin-1 function in mammary gland of domestic animals.

MATERIALS AND METHODS

General reagents and methods

Rabbit polyclonal antibody against N-terminal amino acid sequences 1-105 of Caveolin-1 was bought from Chemicon International Inc. (Temecula, CA). Caveolin-1 (clone 2297), 2 (clone 65) and 3 (clone 26) monoclonal antibodies were purchased from BD Biosciences. Dulbecco's modified Eagle medium (DMEM), α -MEM, F12K nutrient mix medium, penicillin, streptomycin, L-glutamine, fetal bovine serum, horse serum and lipofectamine plus reagent were purchased from Life Technologies (Gaithersburg, MD, USA). Freund's complete and incomplete adjuvants, Hoechst 33342 dye, Texas-Red conjugated phalloidin were obtained from Sigma-Aldrich Fine Chemical, Inc. Normal goat serum and FITC-conjugated donkey anti-rabbit IgG secondary antibody were purchased from Jackson Immunoresearch Laboratories, Inc. PA (USA).

Plasmid construction

Caveolin-1 cDNA was amplified from adult C57BL/6J mouse brains by RT-PCR and sub-cloned into pGEM-T easy vector by TA cloning (Promega Inc. USA). The paired primer sequences used for RT-PCR were as follows: forward primer, CTCGAGATGTCTGGGGGCAAATACG TG; reverse primer, TCTAGATATCTCTTTCTGCGTGCT GATGCG. Caveolin-1 was sequenced and excised from pGEM-T easy vector with *Xho* I and *Xba* I restriction enzymes and then subcloned into the same enzyme digested pcDNA4 sites (Invitrogen Inc. USA). This plasmid was designated pcDNA4-caveolin-1. Caveolin-2 and Caveolin-3 cDNAs were cloned from mammary gland of ICR mouse at lactation day 15 and muscle by RT-PCR. The primer sequences used for RT-PCR were as follows: Caveolin-2 forward primer, GAATTCGGTACCATGGGGCTGGAGA

CCGAGAAGGC: reverse primer. AAGCTTTCTAGAGTC GTGGCTCAGTTGCATGC. Caveolin-3 forward primer. CGGCAGCGGCACG AGTC: reverse primer. CTCCCGCA CCAAGTTTTCCCATCT. Caveolin-2 cDNA was cloned into pGEM-T easy vector by TA cloning. The inserted Caveolin-2 cDNA was then excised and subcloned into pcDNA-4 vector between *Kpn* I and *Xba* I restriction enzyme sites. Caveolin-3 cDNA was then subject to nested PCR using the following of primer pair: forward primer, GGATCCCTCGAGATGATGACCGAAGAGCACACGG: reverse primer. AAGCTTTCTAGAGCCTTCCCTTCGCA GCACCACC. The resulting PCR product was digested by *Xho* I and *Xba* I restriction enzymes. The digested Caveolin-3 cDNA was cloned into pcDNA4. These two plasmids were designated as pcDNA4-caveolin-2 and pcDNA4-caveolin-3, respectively.

Peptide synthesis

A synthetic peptide corresponding to 50-65 of amino acid sequence (DLVNRDPKHLNDDVVK) of human Caveolin-1 (accession number: Hs.74034), purchased from GlycoNex (Taipei, Taiwan), was purified to 90% homogeneity with HPLC and modified by a Multiple Antigenic Peptide (MAP) system. The criterion for choosing immunogenic peptide sequences of Caveolin-1 is illustrated in the text and analyzed using the DNA Star computer program (DNASTAR, Inc.).

Immunization

A 1.5 kg New Zealand semi-lop white rabbit was used to generate the antibody. Pre-serum was collected from the rabbit prior to immunization. At the start of immunization, 1 mg of peptide-MAP was emulsified completely with Freund's complete adjuvant then injected subcutaneously into the rabbit. Triplet booster immunizations were performed through subcutaneous injection of 0.5 mg of peptide-MAP emulsified with Freund's incomplete adjuvant into the rabbit every four weeks following the initial immunization. The rabbit was bled and the titer of anti-Caveolin-1 antibody was determined three weeks after the second booster injection.

Cell culture and transfection

Mouse fibroblast NIH 3T3 (ATCC CRL-1658) and human epithelial A431 (ATCC CRL-1555) were obtained from the American Type Cell Collection (ATCC) and were propagated in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of both penicillin and streptomycin. Human mammary epithelial MCF-7 cells (ATCC HTB-22) were cultured in α -MEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of both penicillin and streptomycin. Rat pituitary adenoma GH3 cells (ATCC CCL-82.1) were propagated in F12K

nutrient mix medium supplemented with 2.5% fetal bovine serum, 15% horse serum, 2 mM L-glutamine, 100 units/ml of both penicillin and streptomycin. Goat mammary epithelial primary cell (GMEC) culture was maintained according to the method of Pantschenko et al. (2000). GMEC cells were maintained in MCDB 171 medium supplemented with Mammary Epithelial Growth Supplement (MEGS; Cascade Biologics Com.).

Transfection of GH3 and MCF-7 cells were carried out using lipofectamine plus reagent according to the manufacturer's protocol. Briefly, the day before transfection, GH3 cells were plated on 35 mm cell culture grade Petri-dishes (Falcon, Inc.) at cell densities of 3×10^5 . DNA (final to 1 μ g) and 6 μ l PlusTM reagent were diluted into 100 μ l serum-free medium. 4 μ l lipofectamine was then added to the 100 μ l serum-free medium, the two pre-complexes mixed and then incubated for 15 min at room temperature. The DNA-PlusTM-lipofectamine reagent complexes were then mixed with 800 μ l fresh serum-free medium and added to the cells in the Petri dishes. Serum-free medium was replaced with fresh complete medium after 3 h. Immunocytochemical analysis and protein extraction were performed 48 h after transfection.

Immunocytofluorescence

A431 and NIH 3T3 cells were grown on 22 \times 22 mm glass cover slips at cell densities of 3×10^5 for one day. Cells were then briefly washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Then the cells were permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 min at room temperature, then immersed in blocking solution with 10% normal goat serum in PBS for 1 h. Further, they were incubated with anti-Caveolin-1 peptide polyclonal antibody (1:300 in PBS) overnight at 4°C. After three washes with PBS, further incubation was done with FITC-conjugated donkey anti-rabbit IgG secondary antibodies (1:300 dilution) for 2 h at room temperature. After another three washes with PBS, cells were incubated with Hoechst 33342 dye (10 ng/ml) and Texas-Red conjugated phalloidin (2.5 μ g/ml) for 10 minutes at room temperature followed by three PBS washes. Slides were mounted with Mowiol 4-88 (Calbiochem, Inc.) and cells were visualized by confocal laser scanning microscopy (LSM 510, Zeiss) and digitally photographed.

Dot blotting and Western immunoblotting

For dot blotting, synthesized Caveolin-1 peptide was serially diluted in 1 μ l of distilled water at concentrations from 10 pg to 1 μ g, then spotted on NC membrane prior to immunoblotting. For Western blotting, cellular proteins were extracted from goat MEC, NIH 3T3, A431, GH3 and pcDNA4-caveolin-1, pcDNA4-caveolin-2 or pcDNA4-

caveolin-3 transfected GH3 or MCF-7 cells with RIPA buffer (50 mM Tris-HCl, pH 7.0, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 56 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM Na₃VO₄, 1 mM NaF, 10 mM Na₂P₂O₇), then centrifuged at 12,000 rpm at 4°C for 10 minutes, and the supernatants collected. The concentration of proteins was quantified by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Twenty µg of each protein sample was denatured in Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris HCl, pH 6.8, 0.01% bromophenol blue) for 5 minutes at 95°C. Proteins were separated on 15% SDS-PAGE and then transferred to PVDF membrane. Transferred protein bands were visualized with Amido-black to verify equal loading of samples. Blots were washed with TBST buffer (120 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) then placed in TBST buffer supplemented with 5% skimmed milk and incubated for 1 h at room temperature. Blots were then incubated with rabbit anti-Caveolin-1 peptide antibody, commercial Caveolin-1, Caveolin-2, Caveolin-3, or pre-immune serum (1:3,000 in TBST) for 24 h at 4°C with gentle agitation. After three washes with TBST, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:3,000, Amersham Biosciences) for 2 h at room temperature and washed again as described previously. Membrane-bound secondary antibodies were detected by ECL Western blotting reagents (Amersham Biosciences) and exposure of the blots to Kodak BioMax MR scientific imaging film.

Immunoprecipitation

The A431 crude protein was extracted with RIPA buffer. Total protein was quantified and diluted to 1 µg per microliter in RIPA buffer in a final reaction volume of 500 µl, then pre-cleaned by incubating with 20 µl of protein A-sepharose slurry (Amersham Biosciences) with agitation for 30 minutes at 4°C, followed by centrifugation 12,000 rpm for 5 minutes at 4°C. The supernatants were immunoprecipitated either with 1 µl pre-immune serum or the third boosted anti-Caveolin-1 peptide antibody for 6 hours at 4°C. Precipitates were obtained by mixing supernatants with 20 µl protein A-sepharose slurry for 2 h, then centrifuged at 12,000 rpm at 4°C for 5 minutes and washed three times with 500 µl RIPA buffer, then suspended in 50 µl of 2× Laemmli buffer, boiled for 5 minutes, and finally subjected to gel electrophoresis and Western blotting as described above.

Immunohistochemistry

Seven µm thick cryo-sections of paired normal and tumorous tissues fixed by 4% paraformaldehyde (including

50 patients, 25 with breast and 25 with colon cancers) were obtained from National Taiwan University Hospital. Each tissue sample comprised normal and tumorous sections from same patient. The normal tissues surrounded tumors were dissected and cryo-sectioned as controls for basal levels of Caveolin-1 expression. The sections were pretreated with 0.3% H₂O₂ for 30 minutes at room temperature, then rinsed twice with PBS and incubated for 1 h at room temperature with 10% normal goat serum. After blocking, sections were then incubated with anti-Caveolin-1 peptide antibody (1:100 PBS dilution) in a humid chamber overnight at 4°C. After three times washes with PBS, the sections were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:300, Amersham Biosciences) for 2 h at room temperature. The peroxidase labeled secondary antibody was detected by diaminobenzidine tetrahydrochloride (DAB) precipitation with an ABC kit (Vectastain Elite) according to the manufacturer's instructions. The expression levels of Caveolin-1 in these sections were categorized according to the timing of DAB precipitation and the intensity of DAB reddish precipitate in sections after peroxidase exposure to H₂O₂ and DAB. The expression levels of Caveolin-1 in these tissue sections were ranked into six grades under light microscope depending on the intensity of brown precipitate formation. Data analysis was performed by SAS software (version 9.1). The difference between normal and tumorous sections in each tissue by pairing T testing.

RESULTS

Anti-Caveolin-1 peptide antibody reacts with Caveolin-1 antigen

A polyclonal anti-Caveolin-1 antibody to detect the N-terminal region of Caveolin-1 expressed in human, goat and mouse was generated. Based on its relative hydrophilicity, surface probability, antigenic index, exposure to the cytoplasm and high conservation in many species, a peptide containing amino acids 50-65 of Caveolin-1 was synthesized and then subcutaneously injected into a rabbit. After the second booster injection of the synthesized peptide into the rabbit, we used a dot blot assay to determine whether this peptide immunogen could raise immunogenicity and whether the resulting antibody could recognize the original Caveolin-1 peptide. Serial dilutions of Caveolin-1 peptide were blotted onto NC membrane and exposed to second boosted anti-Caveolin-1 peptide antibody. The membrane bound antibody was exposed to peroxidase labeled secondary antibody then signals were detected using ECL. The result showed that it was possible to detect 10-100 pg of the peptide antigen using the peptide antibody (Figure 1A). The commercial antibody could detect 100 ng of peptide antiserum. No apparent signal was detected when

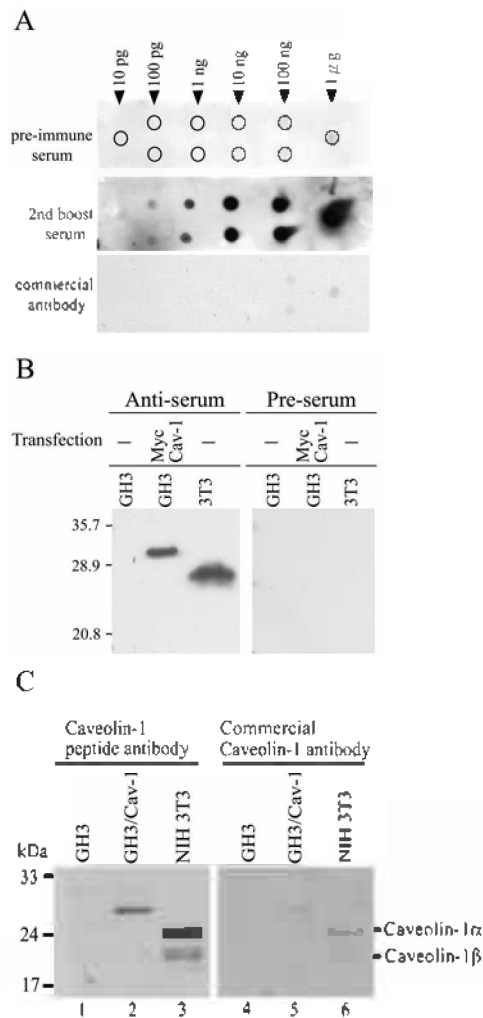


Figure 1. Anti-Caveolin-1 peptide antibody recognizes the recombinant mouse Caveolin-1. (A) Immuno-dot blotting analysis of the peptide antibody titer. The synthesized peptide was serially diluted as indicated and blotted in duplicate onto NC membranes. Diluted (1:3,000) peptide antibody was applied for immuno-dot blotting. Pre-immune serum and a commercial antibody were used as negative and positive controls respectively. (B) Cellular proteins were extracted from GH3, NIH 3T3 cells and Myc-tagged Caveolin-1 transiently transfected GH3 cells, then separated on 12% acrylamide gels and Western blots performed. Pre-immune serum was used as a negative control. (C) Cellular proteins were extracted from NIH 3T3, GH3 and pcDNA4-caveolin-1 transfected GH3 cells. Western blot was performed after these proteins were separated on 15% acrylamide gels using our Caveolin-1 peptide antibody (left panel, 1:3,000 dilution) or commercial Caveolin-1 antibody (right panel, 1:3,000 dilution) as primary antibodies. Same dilution of peroxidase conjugated secondary antibodies (1:3,000 dilution) was used before ECL detection. Left panel was obtained by exposing to X-ray film for 5 seconds where as right panel exposing for 5 minutes.

pre-immune serum was used as primary antiserum (Figure 1A). Although this peptide antibody reacted with amino acids residues 50-65 on Caveolin-1, this didn't mean it

would necessarily recognize full length Caveolin-1. We then addressed whether the anti-Caveolin-1 peptide antibody could recognize full length recombinant Caveolin-1. GH3 cells are anterior pituitary tumor cell line of rat that expresses undetectable levels of endogenous Caveolin-1 protein, hence GH3 cells were considered as an ideal cell line for expressing and detecting the exogenous recombinant Caveolin-1. A plasmid containing C-terminal Myc-tagged Caveolin-1, driven by a cytomegalovirus (CMV) promoter was constructed and transfected into GH3 cells. Cellular protein was extracted 48 hours after transfection. Western blotting was performed using the anti-Caveolin-1 peptide antibody. The peptide antibody not only successfully detected 21-24 kDa protein in NIH 3T3 cells in which endogenous Caveolin-1 (21-24 kDa) is expressed (Engelman et al., 1997) but also the 29 kDa recombinant Caveolin-1 in transfected GH3 cells (Figure 1B). No signal was detected when protein was extracted from non-transfected GH3 cells or when the Caveolin-1 antibody was replaced with pre-immune serum (Figure 1B). We next tried to address whether the generated Caveolin-1 peptide antibody has higher sensitivity in detecting full length Caveolin-1 than its commercial counterpart (Chemicon international Inc. Cat. No. AB3823 and BD Biosciences). For this, the cellular proteins were extracted from pcDNA4-caveolin-1 transfected GH3, GH3 and NIH 3T3 cells. Western blot was performed after using same amount of antibody. The data revealed that the peptide antibody gave an apparent signal in 5 seconds after exposure to X-ray film, while commercial antibody needed 5 minutes to give a weak signal (Figure 1C). Two commercial anti-Caveolin-1 antibodies were tested and got similar results. This result demonstrated that the generated peptide antibody has higher sensitivity and could detect both overexpressed and endogenous Caveolin-1 in cultured cells by Western blotting.

Anti-Caveolin-1 peptide antibody possesses high specificity and reacts with endogenous Caveolin-1 from mouse, goat and human cells by Western blotting

Caveolin family has three genes, Caveolin-1 and Caveolin-2 are ubiquitously expressed, while Caveolin-3 is specifically expressed in muscle (Song et al., 1996b; Smart et al., 1999). To investigate whether the Caveolin-1 peptide antibody cross-react with other Caveolin proteins, Caveolin-2 and Caveolin-3 cDNAs were amplified from mouse mammary gland and muscle by RT/PCR. Both of the cDNAs were cloned into mammalian expression vector, pcDNA4. All of three Caveolin genes were separately transfected into MCF-7 cells which lack Caveolin-1 and Caveolin-2 expression (Park et al., 2002) and Western blotting was performed with different Caveolin antibodies. The result indicated that Caveolin-1 peptide antibody

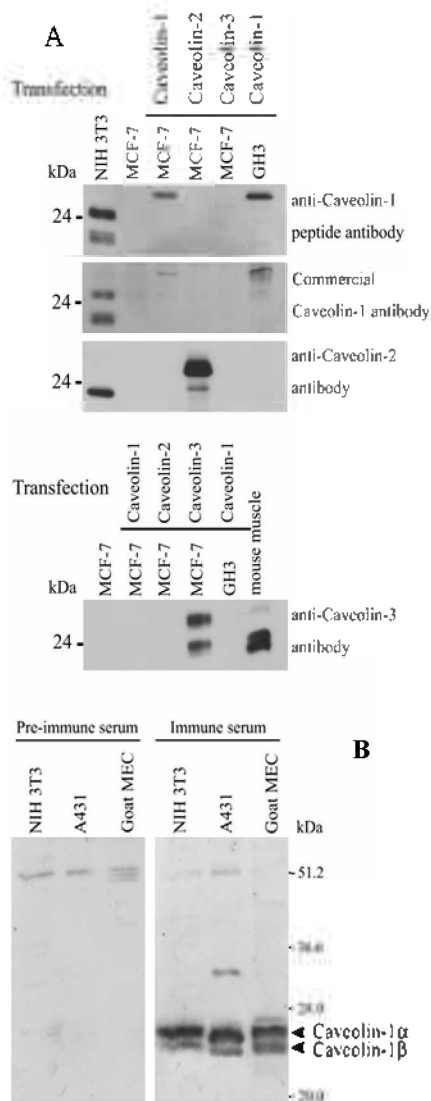


Figure 2. Anti-Caveolin-1 peptide antibody specifically recognizes Caveolin-1 and reacts with endogenous Caveolin-1 from mouse, goat and human cells by Western blotting. (A) All the three Caveolin cDNAs were separately transfected into MCF-7 cells, Western blot was performed using our Caveolin-1 peptide antibody, commercial anti-Caveolin-1 antibody, Caveolin-2 (up-panel) or Caveolin-3 antibody (lower panel) as indicated. Cellular proteins extracted from Caveolin-1 transfected GH3, NIH 3T3 and mouse muscle cells were used as positive controls for detecting Caveolin-1, Caveolin-2 and Caveolin-3. (B) Anti-Caveolin-1 peptide antibody reacted with endogenous Caveolin-1 extracted from different species. Cellular proteins were isolated from mouse NIH 3T3, human A431 cells and goat primary mammary epithelial cells. Proteins were then separated on 10% acrylamide SDS denaturing gel and subjected to Western blotting using the anti-Caveolin-1 peptide antibody. Pre-immune serum was used as a negative control (left panel). The arrows indicate Caveolin-1 expressed in cells.

specifically reacted with Caveolin-1 and do not cross-react with Caveolin-2 and Caveolin-3 over-expressed in MCF-7

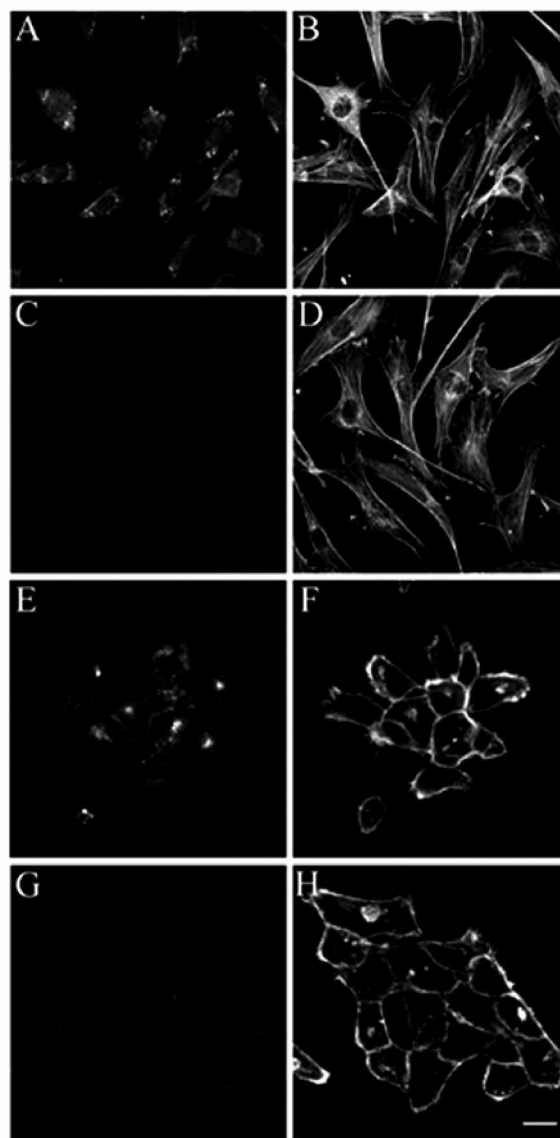


Figure 3. Immunofluorescent staining of endogenous Caveolin-1 in NIH 3T3 (A-D) and A431 (E-H) cells. (A) Mouse NIH 3T3 and (E) human A431 cells were positively stained for Caveolin-1 using the peptide antibody and anti-rabbit IgG-FITC conjugated secondary antibody. Rabbit pre-immune serum was used as a negative staining control (C, G) for the peptide serum. Cellular morphology was observed using phalloidin conjugated with Texas-Red to stain filamentary actin in cells (B, D, F and H). Scale bar is 20 μ m.

cells (Figure 2A). Cellular proteins extracted from Caveolin-1 transfected GH3, NIH 3T3 and mouse muscle cells were used as positive controls for detecting the Caveolin-1, Caveolin-2 and Caveolin-3 protein, respectively (Figure 2A). The amino acid sequence of the immunogenic Caveolin-1 peptide chosen for this study is conserved among human, bovine, goat (accession number: DQ912799), rat and mouse. Therefore the anti-Caveolin-1 peptide antibody should be capable of detecting Caveolin-1

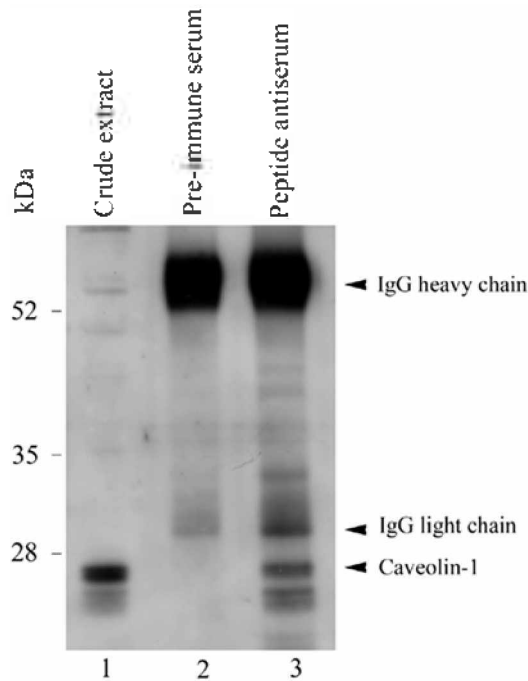


Figure 4. Immunoprecipitation of Caveolin-1 from A431 cells by anti-Caveolin-1 peptide antibody. Total protein was extracted from A431 cells and immunoprecipitated with the anti-Caveolin-1 peptide antibody (lane 3) and its pre-immune serum (lane 2). The immunoprecipitated proteins and A431 crude protein extract (lane 1) were then separated on 10% SDS-PAGE and Western blotted using the anti-Caveolin-1 peptide antibody as the primary antibody.

expressed in human, goat and mouse cells. To verify whether the peptide antibody could react with Caveolin-1 from these species. Western blotting using the anti-Caveolin-1 peptide antibody was performed on cellular proteins extracted from mouse (NIH 3T3), human (A431) and goat primary mammary epithelial cells (GMEC). NIH 3T3, A431 and mammary epithelial cells have been documented to express endogenous Caveolin-1 (Koleske et al., 1995; Couet et al., 1997; Park et al., 2002). A 24 kDa intensive positive band belonging to Caveolin-1 α and a 21 kDa band belonging to Caveolin-1 β were detected in cellular protein extracted from all species (Figure 2B, right panel). A 25 kDa faint band was found in goat MEC cellular protein and, based on its molecular weight being slightly higher than 24 kDa, was suspected as a post-translationally modified Caveolin-1. No signal was detected when pre-immune serum was used for Western blotting (Figure 2B, left panel). A 34 kDa signal was detected in human cells; whether this signal belongs to Caveolin needs further exploration.

Endogenous Caveolin-1 was detected by anti-Caveolin-1 peptide antibody after immunocytofluorescent staining

We then determined if our peptide Caveolin-1 antibody

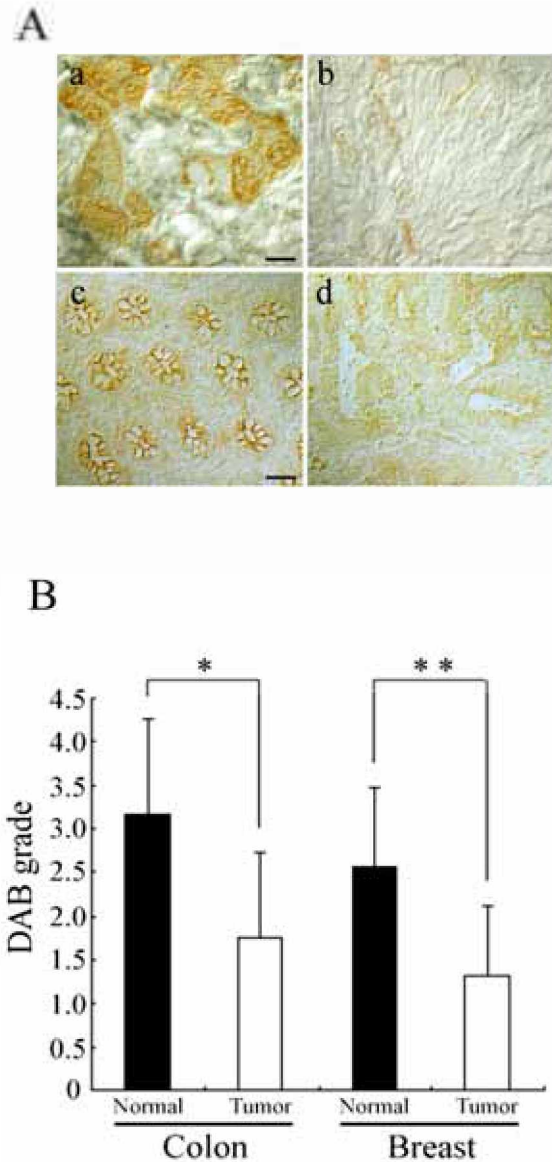


Figure 5. Reduced levels of Caveolin-1 expression were detected with the anti-Caveolin-1 peptide antibody in tumorous breast and colon tissue. (A) Twenty-five pairs of breast (a, b) and colon (c, d) tissues were chosen for an analysis of changes in Caveolin-1 expression with the anti-Caveolin-1 peptide antibody in both normal (a, c) and tumorous (b, d) tissues and observed by immunohistochemical staining. Peroxidase-conjugated anti-rabbit IgG secondary antibody was used and detected by using a VECTASTAIN[®] ABC kit. The normal and malignant tumorous tissues in each paired experiment were from the same individual. Scale Bar = 100 μ m. (B) Statistical analysis of Caveolin-1 expression levels in normal and tumorous tissues. Twenty-five pairs of breast and colon tissues each were collected and performed immunohistochemical staining, the expression levels of Caveolin-1 in these tissue sections were ranked into six grades under light microscope depending on the intensity of brown precipitate formation. SAS (statistical analysis software) was employed for statistical analysis ($p < 0.01$). Bars represent the standard errors.

can recognize endogenous Caveolin-1 in mouse and human cells by immunocytofluorescent assay. For this NIH 3T3 (Figure 3A-D) and A431 (Figure 3E-H) cells expressing endogenous Caveolin-1 were used. A punctate pattern of Caveolin-1 was detected in NIH 3T3 (Figure 3A) and A431 cells (Figure 3E) using our peptide antibody; but no signal was shown when pre-immune serum was used instead of the peptide antibody (Figure 3C and 3G). This result indicated that our peptide antibody could be used for immunocytochemical detection of native Caveolin-1 in cultured cells and this led us to test this peptide antibody in further immunoprecipitation experiment. A431 crude protein extract was immunoprecipitated either with pre-immune serum or third boosted anti-Caveolin-1 peptide antibody and then subjected to Western blotting with the peptide antibody (Figure 4). Endogenous Caveolin-1 was successfully immunoprecipitated from A431 cellular protein by the peptide antibody. The precipitated Caveolin-1 showed identical protein mass compared with Caveolin-1 in A431 crude extract (Figure 4). The pre-immune serum did not precipitate any protein recognized by the anti-Caveolin-1 peptide antibody.

Down-regulated of Caveolin-1 expression in tumorous breast and colon tissues was detected by the peptide antibody

Loss or reduced expression of Caveolin-1, linked with the progression of breast and colon cancers, has been regarded as a marker for the progression of these cancers (Sloan et al., 2004; Wikman et al., 2004). Caveolin-1 was detected using the peptide antibody in normal human breast (Figure 5A-a) and colon (Figure 5A-c) tissues by immunohistochemical staining. Using same strategy, attempts were made to detect and see whether Caveolin-1 is down regulated in tumorous breast and colon tissues. The result showed decrease expression of Caveolin-1 in those tissues (Figure 5A-b and d). Further, comparison of the different expression level of Caveolin-1 between normal and tumorous tissues using more samples was done. Twenty-five samples each of the normal and cancerous tissues were obtained from female patients suffering from breast and colon cancer. Frozen sections of normal and tumorous tissues of the same patients were subjected to immunohistochemical staining using the anti-Caveolin-1 peptide antibody as the primary antibody. Caveolin-1 expressions in these sections were detected by peroxidase conjugated anti-rabbit IgG secondary antibody. The immobilized peroxidase was then reacted with its substrate H_2O_2 and DAB forming brown colorimetric precipitate. The levels of Caveolin-1 expression were categorized according to the timing of brown precipitate formation and the intensity of precipitates in tissue sections. We ranked the expression intensity of Caveolin-1 into six grades under

light microscope. A significant reduction in Caveolin-1 expression was observed in tumorous breast and colon cancer tissues compared to their normal tissues (Figure 5B). This result demonstrates that this anti-Caveolin-1 peptide antibody can immunohistochemically detect endogenous Caveolin-1 and be used in detecting the progression of breast and colon cancers.

DISCUSSION

In this study, we validated that generated rabbit polyclonal anti-Caveolin-1 peptide antibody could detect recombinant and endogenous Caveolin-1 expressed in mouse, goat and human. This result suggests at least one immunogenic epitope exists in amino-acid residues 50-65 of Caveolin-1 and the epitope is conserved among mouse, goat and human. On the basis of the result show this polyclonal antibody could be extensively used for detecting Caveolin-1 in these animals.

Dot blotting data and Western blotting revealed our peptide antibody could react with the Caveolin-1 peptide antigen and was more sensitive than some commercial antibodies. The commercial rabbit anti-Caveolin-1 polyclonal antibody (Chemicon International Inc. Cat. No. AB3823) was generated using amino acid residues 1-101 of human Caveolin-1 as immunogen. Hence, the commercial antibody might recognize more epitopes expanding on 1-101 residues than on residues 50-65 of Caveolin-1.

Caveolin-2 and Caveolin-3 share 38% and 65% identity in amino acid sequences to Caveolin-1, respectively (Smart et al., 1999). There is 69% divergence in amino acid residues in the 50-65 region between Caveolin-1 and Caveolin-2. Caveolin-3 has 28.6% divergence to Caveolin-1 in the same region. We demonstrated that the Caveolin-1 peptide antibody specifically recognizes both overexpressed and endogenous Caveolin-1, but not Caveolin-2 or Caveolin-3. On the basis that endogenous Caveolin-3 is an 18 kDa protein and specifically expressed in muscle (Song et al., 1996b), the 21-24 kDa protein masses detected from NIH 3T3, A431 and goat mammary epithelial cells in this study, should be Caveolin-1 α and Caveolin-1 β .

Caveolin-1 attaches to the cell membrane through its central domain (residues 102-134) with its N- and C-termini oriented towards the cytoplasm. N-terminal (residues 82-101) is necessary for Caveolin mutually oligomerization and signal molecule interactions. Caveolin-1's C-terminal domain (residues 135-178) is required for membrane attachment and protein-protein interaction (Krajewska and Maslowska, 2004). Hence, amino acid residues 82-178 of Caveolin-1 bind its interactive proteins or lipids in cells. The residues 50-65 of Caveolin-1 chosen as immunogen were free and might not interfere the binding with its interactive protein. Our peptide antibody was

successfully immunoprecipitated with endogenous Caveolin-1 from A431 cells. Based on these results, we conclude that the anti-Caveolin-1 peptide antibody may be used for further studies on protein-protein interaction by co-immunoprecipitation analysis.

Down-regulation of Caveolin-1 through point mutation (Joo et al., 2004), chromosome deletion and promoter methylation (Engelman et al., 1998; Cui et al., 2001; Chen et al., 2004) has been documented in many cancers, including breast cancer (Chen et al., 2004), colon carcinomas (Zenklusen et al., 1995), colorectal cancer (Lin et al., 2004) and small cell lung cancer (SCLC) (Sunaga et al., 2004). In this study, decreasing Caveolin-1 expression was consistently observed in colon and breast cancerous cells. Thus, our peptide antibody can be used for immunohistochemical staining. A panel of antibodies raised against various domains of Caveolin-1 was tested to detect distinct subcellular pools of Caveolin-1 protein in MDCK cells. It showed that each antibody varied in their ability to label Caveolin-1 located in cells. The epitopes of Caveolin-1 detected by each antibody are highly depending on fixation method (Bush et al., 2006). This study reminds that we need to investigate the location of labeled Caveolin-1 in cells by our antibody systematically to check the immunostaining results using different fixation methods.

In summary, in this study we have produced an anti-Caveolin-1 peptide antibody that can detect Caveolin-1 proteins either extracted from mouse, goat and human cells using Western blotting, or immunohistochemical analysis and immunoprecipitation. We also demonstrated that this anti-Caveolin-1 antibody could detect decreased levels of Caveolin-1 in tumorous breast and colon tissue sections compared to normal tissues.

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