ブタ大動脈平滑筋に存在する190キロダルトンタンパク質の免疫化学的研究

Mem.Muroran Inst. Tech., 53 (2003) 107~114

Immunochemical Characterization of 190K Protein from Porcine Aorta Smooth Muscle

Li-Juan YAN*, Sachiko HATSUKAIWA*, Tomoko HONDA*,
Naoki YOSHINAGA*, Naoki NIIDA* and Yoh OKAMOTO*

(Received 6 May 2003, Accepted 29 August 2003)

Contractility of muscle cell is established by mutual sliding of two types of protein polymers. Myosin ATPase and actin are the protomers of these filamentous oligomers. Sarcomere is a contractile unit machinery in striated muscle. However, there is no such unit structure in smooth muscle. During the studies on unconventional myosin, we have found a 190K actin binding protein from aorta smooth muscle. This protein has been identified as IQGAP1, an actin binding protein in non-muscle cells. In order to find out the physiological role of IQGAP1 in smooth muscle, specific antibody has been prepared. As a first step of the application, the amounts of IQGAP1 in aorta media smooth muscle has been estimated immunochemically. Further studies on possible IQGAP1 related protein has been described.

Key words : Actin binding, Smooth muscle, IQGAP1

1 INTRODUCTION

Contractile apparatus of smooth muscle cell appears to be quite different from those of striated muscle. Even through there is no sarcomere like motile unit structure within the smooth muscle, the fundamental mechanism of smooth muscle contraction is supposed to follow sliding filament mechanism proposed for striated muscle. The contractile unit structure and the mechanism of molecular assembly are not well known in spite of well documented stabilization of myosin filament through the light chain phosphorylation *in vitro*. Calcium and calmodulin dependent phosphorylation of myosin light chain activates actomyosin ATPase resulting in contraction. However, the contractility and the extents of light chain phosphorylation is not always to be proportional to the intra-cellular Ca^{2+} concentration. [1,2] GTP-dependent contractility at moderate Ca^{2+} concentration has been reported to explain this fact.[3,4] Rho and its effectors dependent process has been proposed for the regulation of myosin light chain phosphorylation.[5] This mechanism also appears to be operated in non-muscle cells for the motility. Particularly, alignment of actin filament must be essential for the integration of contractile force. The actin filament in smooth muscle also appears to exist in less ordered structure such as dense body. Filament formation of myosin and actin and the stability might be much more dynamic nature than that of striated muscle. The regulatory mechanism for proper alignment of contractile proteins is not well known. Another striking property of vascular smooth muscle is a potential to transform to migratory synthetic cell type. At this point of view, smooth muscle cell has properties similar to those of non-muscle. In fact, myosin Ic, a single headed unconventional myosin, was identified in aorta smooth muscle. [6] During the studies on myosin Ic, we have found another calmodulin related 190kDa protein. This protein binds to F-actin in an ATP independent manner. Both the molecular weight and the partial amino acid sequence indicated a high homology to those of human

* Department of Applied Chemistry

brain IQGAP1, a small GTPase and F-actin binding protein contributing actin reorganization and cell-cell adhesion. [7] Immunochemical analysis using anti-human IQGAP1 antibody also indicated a strong cross-reactivity with the 190kDa protein. This is a first report of IQGAP1 exists in muscle cell. We have raised polyclonal antibody against the aorta IQGAP1 for further investigation of the physiological roles in contractile cell in such cases as quantitation, subcellular localization and screening the binding partner in the cell. The presence of IQGAP1 in aorta smooth muscle suggests contributions for cellular processes such as actin reorganization during contraction-relaxation cycle, association of cytoskeletal structure to cell membrane.

2 MATERIALS AND METHODS

2.1 Chemicals

Q-Sepharose FF and SP-SepharoseFF ion exchange resins, Sepharose CL4B resins were purchased from Amersham Bioscience. ATP were from Roche Molecular Biochemicals. Anti IQGAP1 antibodies were purchased from Santa Cruz Biotechnology and Becton Dickinson Inc. EGTA were purchased from Sigma. Phenylmethylsulfonyl fluoride (PMSF) and diisopropyl phosphofluoridate (DFP) were purchased from Wako Chemical Industry. Other chemicals were of analytical grade.

2.2 Protein Determination

Protein concentrations were determined either by Pierce BCA assay or by biuret method.

2.3 SDS poly acrylamide gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli . [8]

2.4 Proteins

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt.[9] Calmodulin was prepared from scallop testis according to the method of Yazawa et al. [10] Calmodulin has been either prepared from Scallop testis or purchased bovine brain one from Sigma Chem. Co. Aorta 190kDa protein was prepared from porcine aorta media smooth muscle following the procedure for myosin I preparation. [6]

2.5 F-actin Binding Assay

Binding of F-actin to 190K protein was measured by co-sedimentation assay as described. [6]

2.6 Antibody Preparation

190kDa was emulsified with Freund's complete adjuvant (1:1, vol/vol) before immunization. A rabbit was injected intradermaly with the emulsified immunogen mixture. For boost immunizations, 1mg of the 190K protein that was emulsified with Freund's incomplete adjuvant was injected three times with four weeks interval, and the antiserum was collected regularly after the final immunization.

2.7 ELISA and Immuno Blotting

Samples were electrophoresed on 12% gel in the presence of SDS, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 10% (w/v) skim milk, 0.5M NaCl, 20mM Tris-HCl (pH7.4), and 0.05% Tween20 (Solution B) at 4℃ overnight, the PVDF membrane was incubated with anti-190K protein antibody that was diluted in solution B containing 1% bovine serum albumin at room temperature for 2 hour. Binding of the primary antibody was detected with horseradish peroxidase conjugated anti-rabbit IgG. Color development was done with 2,4-dichloro-1-naphtol and N,N-dimethyl-p-phenylendiamine sulfate. ELISA, enzyme linked immunosorbent assay, was done as has been described .

3 RESULTS AND DISCUSSION

3.1 Isolation of 190K from porcine aorta smooth muscle

The 190K protein has been recognized as co-purified protein with myosin I, an unconventional myosin, a single headed molecular motor with membrane binding short tail. We developed a procedure to purify 190K protein for the characterization in order to find out the physiolgical role in smooth muscle cell. It has been achieved by sequential chromatography of cation exchange, size exclusion, and anion exchange resins. Initially, the high salt extract in 1mM EGTA, 1mM $MgCl_2$, 5% sucrose, 1mM ATP, 20mM Tris Cl (pH 7.5), 0.1mM PMSF, 0.1 mM DFP from minced aorta has been mixed with SP-Sepharose resin in order to adsorb 190K protein following enrichment by high salt elution. The crude 190K protein fraction has been separated by size exclusion chromatography (Sepharose CL 4B). The pooled fraction confirming 190K protein then been diluted to decrease the ionic strength for further anion exchange chromatography. The 190K protein could be enriched and purified at the Q-Sepharose anion exchange chromatography during shallow salt gradient elution. The 190K protein has been eluted at ~ 0.25 M NaCl during the gradient elution from 0.2 to 0.45M NaCl. The 190K protein could be separated from myosin I at this step as shown in Fig.1.

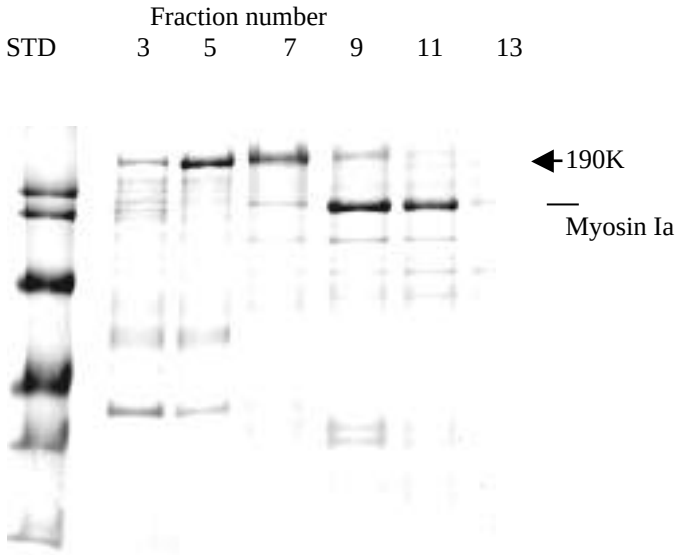


Fig.1 Q-Sepharose column chromatography of aorta 190K protein
A linear salt gradient was developed as described in "MATERIALS and METHODS".
Fractions: 5~7, 190K protein; 9~11, myosin Ia
190K protein was eluted at ~0.25M NaCl

3.2 Binding of 190K protein to F-actin

As a first step to elucidate the role of 190K protein for smooth muscle contractility, F-actin has been added to 190K protein to test their binding, the 190K protein alone could not be spun down under the centrifugal condition for F-actin pelletting at the bottom as shown in Fig.2. In the presence of F-actin, most of 190K protein was found in the precipitate after ultra centrifugation. This shows the binding of 190K protein to F-actin. Furthermore, the binding could also be observed in the presence of Mg ATP. It is therefore, aorta 190K protein appears to be a F-actin binding protein but not to be a family of myosin molecular motor

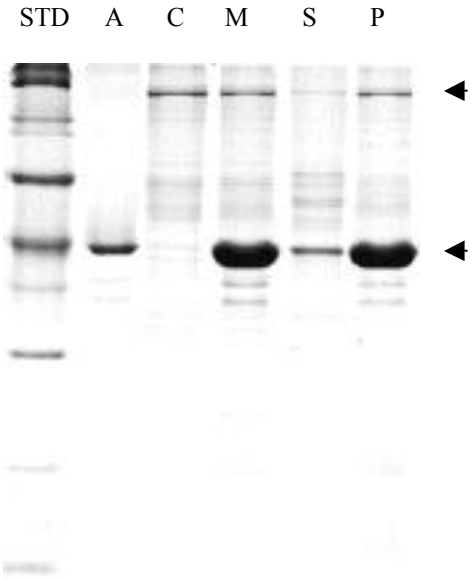


Fig. 2 F-actin binding of aorta 190K protein
A, rabbit skeletal F-actin; C, Q-Sepharose purified 190K protein;M, mixture of A and C; S and P, supernatant and precipitate after centrifugation of M, respectively.

3.3 Identification of aorta 190K protein as IQGAP 1.

To identify the F-actin binding 190K protein found in aorta smooth muscle, we have examined the internal amino acid sequence of 190K protein. In order to achieve higher quality of sequence data, the 190K protein was extracted from gel after SDS-PAGE of column purified 190K fraction. This was effectively done using pre-labelling of 190K protein with a thiol directed fluorescent probe following extraction of fluorescent 190K protein without fixative staining, as shown in Fig.4. The extracted 190K protein has been cleaved proteolytically and seperated in SDS-PAGE. The fragments were blotted on to PVDF membrane and were sequenced. We found a exact match of these sequence with that of IQGAP1 using BLAST on the web as shown in Fig. 3.

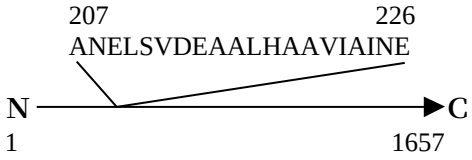


Fig. 3 Partial amino acid sequence of aorta 190K protein exists in human brain IQGAP1

3.4 Calmodulin binding to aorta 190K protein.

IQGAP 1 has been known as a calmodulin binding protein as well as a microfilament binding property. In order to test the properties of 190K protein as IQGAP1, binding of 190K protein to calmodulin-Sepharose resin in the presence or absence of calcium ion has been confirmed. (data not shown)

3.5 Rasing antibody against aorta IQGAP 1 and its utilization for the quantitation in aorta smooth muscle

It is keen to detect and quantitate aorta IQGAP1 for finding the biological function. Polyclonal antibody has been raised in rabbit by intradermal injection of aorta IQGAP1. The antigen has been extracted from SDS-PAGE gel without fixative CBB staining. For this propose, aorta IQGAP 1 soon after Q-Sepharose purification was fluorescently labeled prior to SDS PAGE. The fluorescent band of aorta IQGAP1 has been cut out and extract IQGAP1 with PBS overnight at 4℃ for four times as shown in Fig.4. The overall recovery of IQGAP1 from SDSPAGE gel was ~80%.

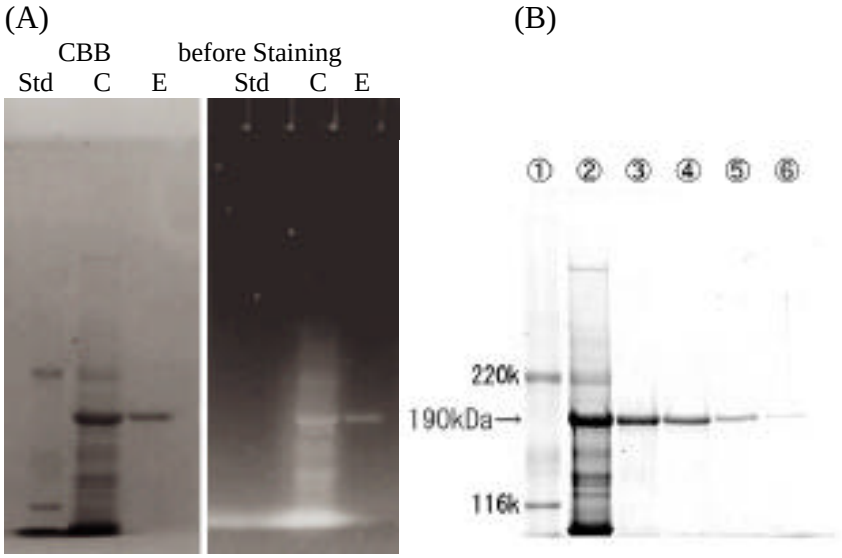
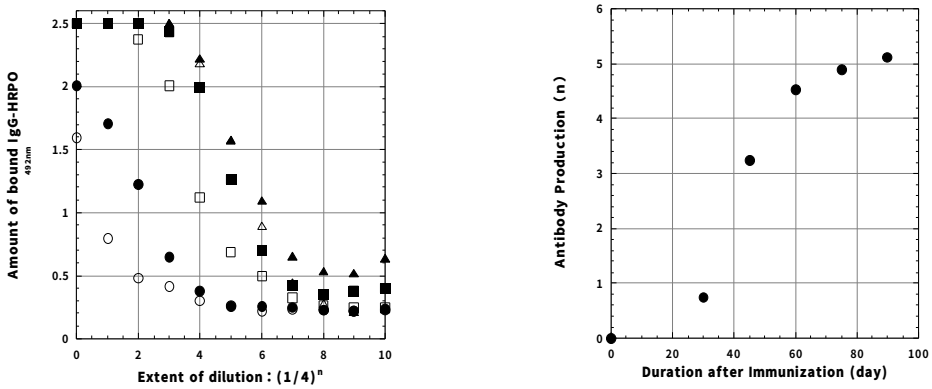


Fig. 4 Extraction of fluorescent aorta IQGAP1 from SDSPAGE (A) Crude IQGAP1 as C; Extracted IQGAP1 as E; (B) time course of extraction, ②as C in (A), ③～⑥ for 1st～4 th day extracts.

Purified and SDS denatured IQGAP1 was emulsified with adjuvant (1:1,vol/vol) before immunization. A rabbit was injected at each hind quadricep, and additionally over both shoulders for three times every 4 weeks. Rabbit serum has been taken before in between and after these injections and were examined the antibody production by both enzyme linked immuno adsorbent assay (ELISA) and western blotting as shown in Fig.5 and 7. Immunization using native 190K following conventional method was unsuccessful. (data not shown)



(A) (B)

Fig. 5 Time cource of anti-aorta IQGAP1 antibody production during immunization
(A) ELISA at each immnization periods (weeks), ○(0), ●(4), □(6), ■(8), △(10), ▲(12);
(B) Time course of specific antibody production

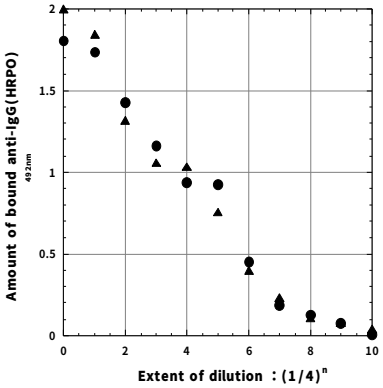


Fig. 6 Anti-aorta IQGAP1 recognize equally both native(▲) and SDS denatured(●) IQGAP1.

A linear correlation between the amount of IQGAP1 and the integrated peak area enable us to determine the amount of IQGAP1 present in aorta tissue as ~1.5 mg in 250g of tissue. Furthermore, we have found another 90kDa protein recognized by anti IQGAP1 antibody. This 90kDa protein, however, did not co-purified with 190K IQGAP1 as shown in Fig.8.

The anti IQGAP1 antibody can be used for the antigen determination at 4000fold dilution. The antibody recognize aorta IQGAP1 equally at both native and denatured conformations as shown in Fig.6.

3.6 Immunochemical characterization of IQGAP1 in aorta smooth muscle.

As a first step for finding physiological function of IQGAP1 in aorta smooth muscle, the amounts in tissue has been determined using calibrated immuno blotting. Calibration curve has been made using highest purify of aorta IQGAP1 as has been prepared acording to the method described in previous section. A set of densitogram was taken using known amounts of IQGAP1 as shown in Fig.7.

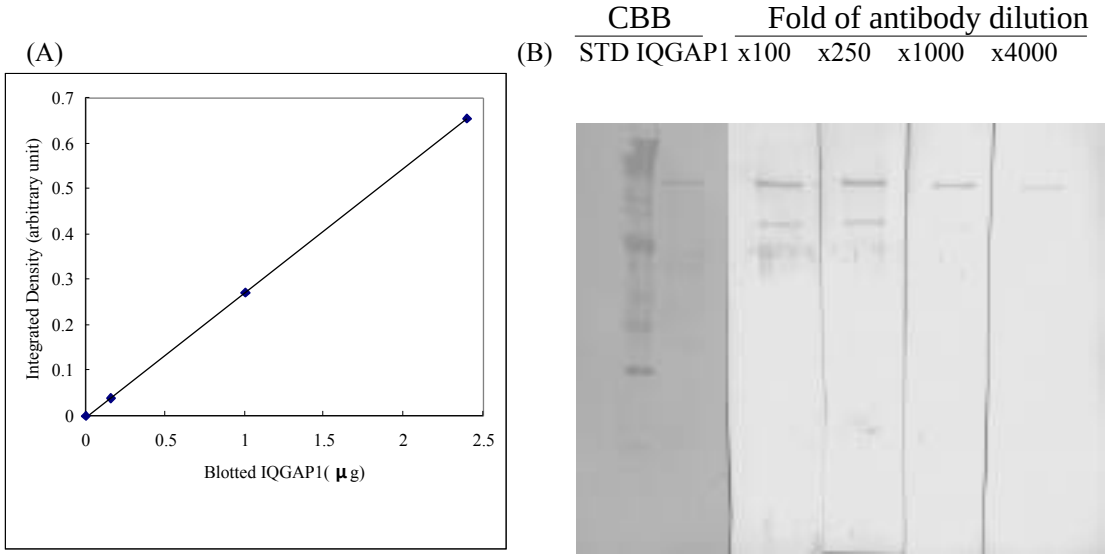


Fig.7 Quantitation of aorta IQGAP1 at micro gram quantities
(A) Densitograms of IQGAP1 immunoblot are proportional to the amounts of protein applied.
(B) Sensitivity of immuno detection determined by serial dilution of antibody on western blot

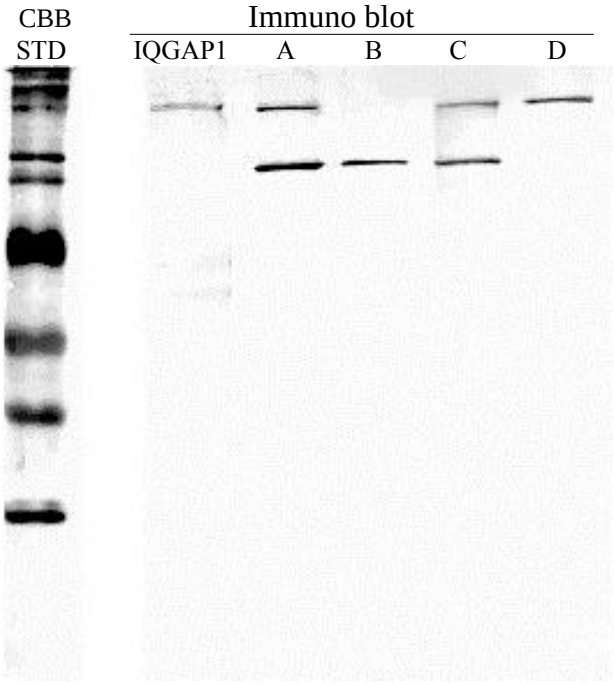


Fig.8 Quantitation of IQGAP1 in aorta media smooth muscle
and a indication of IQGAP1 related 90kDa protein
A, SP-Sepharose fraction; B and C, 55% ammonium sulfate supernatant
and precipitate, respectively; D, Sepharose 4BCL fraction

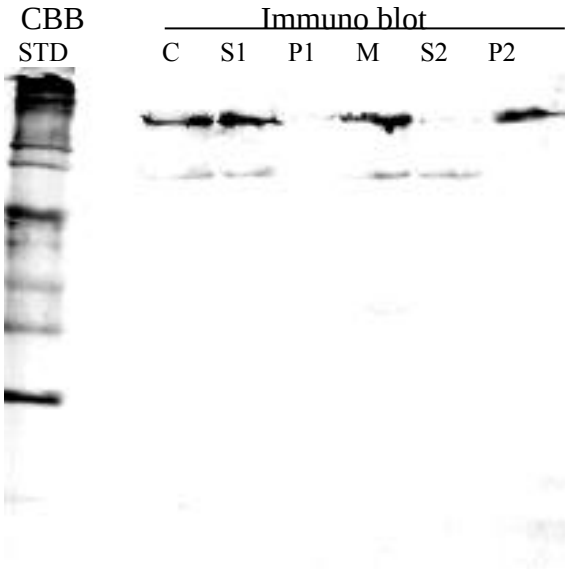


Fig. 9 IQGAP1 related 90kDa protein is not a F-actin binding protein
C, mixture of IQGAP1 and 90kDa protein; S1 and P1, supernatant and precipitate after centrifugation of C, respectively; M, C plus F-actin,S2 and P2, supernatant and precipitate after centrifugation of M, respectively

It is interesting to see if the 90kDa protein bind to F-actin. This was examined by ultracentrifugation of a fraction containing both 190K and 90K protein with or without F-actin. The data clearly showed that 90K protein could not bind to F-actin as shown in Fig.9. Therefore, 90K protein appears to have a common epitope with that of aorta IQGAP1 except F-actin binding site. It is possible to estimate that the 90Kprotein might be similar to carboxy terminal half of IQGAP1 since the actin binding domain locate at the amino terminal end. It is of interesting to see if the 90K protein contains GAP related domain since similar protein has been reported.

Contractile mechanism of smooth muscle seems to be complex. Intracellular alignment of contractile protein within smooth muscle cell appears to be irregular if we compare to see those of striated muscle cell. There is no sarcomere like regularity in the positions of myosin or actin. This striking difference between the two types of muscle cell remind us to think how smooth muscle contractile apparatus has been made for the integration of molecular power strokes, even though it might be a temporal structure.

It is accepted that fundamental mechanisms of contraction are common for striated and smooth muscle. In spite of common concept of mutual sliding between actin and myosin necessary for contraction, the architectures of the contractile apparatus are distinct with each type of muscle. In the case of smooth muscle, there is no sarcomere like unit structure within the cell. Moreover, phosphorylation of the myosin light chain regulates the filament formation upon the activation by intracellular Ca^{2+} increase. This calcium regulatory mechanism is further modulated by GTP dependent amplification. On the other hand, vascular smooth muscle cell is known to undergo transformation the contractile cell type to migratory synthetic one in the case of endothelial cell damage. This might have implied an existence of unconventional myosin necessary for the cell migration as well known in non-muscle cell motility. As a result for first step to examine this possibility, we have identified myosin Ic from porcine aorta smooth muscle. [6] The subcellular localization in A10 cell have shown a diverse distribution including some ten percent in the cytosol. [11] During the course of this study, we have found another calmodulin related 190K protein.

In this report, we have shown the purification method and the binding affinity with calmodulin, F-actin. The partial amino acid sequence indicates a close similarity to those of human brain IQGAP1. All of these data indicate that aorta 190K protein is IQGAP1 identified in muscle for the first time. It should be noted that the yield of aorta 190K protein from tissue is comparable with that reported for that of IQGAP1 from adrenal gland. [12] At present, the exact function of IQGAP1 in vascular muscle cell is not known. Several pioneer works [13-15] about IQGAP1 in non-muscle cells suggest the functions as a cytoskeletal reorganizer concerning the migration and cell-cell adhesion. It is interesting to see if corresponding functions exist in vascular smooth muscle cell. Particularly, contribution of crosslinking actin filaments by IQGAP1 is attractive possibility involved in the contraction-relaxation mechanism and its regulation. Slow tension development and its maintenance of smooth muscle could be influenced by

such contribution. In any case, it is keen to clarify the regulatory mechanism of binding between IQGAP1 and F-actin modulated by cdc42, guanine nucleotides, calcium and calmodulin. It might be critical information to find out the physiological significance of IQGAP1 in vascular smooth muscle cell.

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ブタ大動脈平滑筋に存在する 190 キロダルトンタンパク質の免疫化学的研究

厳麗娟*、甘日岩幸子*、本田智子*、吉永直樹*、新井田直樹*、岡本洋*

平滑筋は横紋筋と異なり発生張力の効率的集積を実現する収縮単位構造サルコメアが存在しないので、強力な収縮力発生の分子像が描けていない。血管平滑筋細胞は動脈硬化発症に際し内皮細胞の損傷などを契機として収縮型形質から遊走性合成型形質へと転換する。我々は細胞遊走性に必要な生体膜結合性ミオシンを探索中にアクチン結合性を示す 190K タンパク質を見つけた。その一次構造などから筋細胞では存在の知られていなかった IQGAP1 というアクチン細胞骨格の再構築や細胞接着に関与するタンパク質であることが判明した。その機能解明に必須な特異抗体の調製とその性能、およびこれを用いた血管平滑筋における IQGAP1 の存在量の推定結果を示した。

キーワード：アクチン結合性、平滑筋、IQGAP1

＊　応用化学科