Vascular Smooth Muscle Calponin
A Novel Troponin T-like Protein

KATSUHITO TAKAHASHI, KUNIO HIWADA, AND TATSUO KOKUBU

SUMMARY In a search for additional Ca$^{2+}$ regulatory components in vascular smooth muscle, a novel troponin T–like protein was purified from bovine aorta smooth muscle. The isolated protein was separated into several isoforms on isoelectric focusing. The major isoelectric variants were focused in the pH region of 8.4 to 9.1. The protein had slightly different molecular masses in the $M_r$ range of 35,000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Its molar ratio relative to tropomyosin in the muscle extract was estimated to be 0.9:1.0. The novel protein bound to the immobilized calmodulin and exhibited a number of common physicochemical properties with gizzard ($M_r = 34,000$) calmodulin-binding and F-actin-binding protein. The aorta and gizzard proteins were immunologically cross-reactive. Both proteins shared a common antigenic determinant with COOH-terminal segments of rabbit skeletal and bovine cardiac troponin T and bound to the immobilized smooth muscle tropomyosin. Both proteins interacted with rabbit skeletal troponin C in the presence and absence of Ca$^{2+}$, but they did not interact with troponin I. These results suggest that the novel protein, which is designated calponin, may be a specialized component of smooth muscle thin filament involved in the regulation of contractile apparatus. (Hypertension 11: 620–626, 1988)

KEY WORDS • vascular smooth muscle • troponin T • tropomyosin • calmodulin-binding protein • calcium

The contractile process of the muscle is regulated by changes in the sarcoplasmic Ca$^{2+}$ concentration. In cardiac and skeletal muscles, actin-myosin interaction is regulated by a specific actin-linked mechanism, the troponin-tropomyosin system. Recent studies have suggested that the contraction of vascular smooth muscle is regulated by a Ca$^{2+}$-dependent mechanism in addition to myosin light chain phosphorylation. The maintenance of vascular smooth muscle tone characterized by a prolonged force production with low energy cost (latch contraction) could be a major contributor to peripheral vascular resistance. To understand potential myogenic factors responsible for the hypertensive state, the regulatory protein (or proteins) in the second Ca$^{2+}$-dependent mechanism must be identified.

We have reported novel proteins with $M_r$ values of 33,000 to 35,000 that are present in large amounts in a variety of vertebrate smooth muscles. The 34,000 $M_r$ protein purified from chicken gizzard smooth muscle is present in equimolar concentration to tropomyosin and interacts in vitro with calmodulin and actin-tropomyosin filament. In the present study, we purified 35,000 $M_r$ protein from bovine aorta smooth muscle and studied immunological and biochemical relationships between the novel protein and troponin T. Based on the present results in conjunction with our observation concerning Ca$^{2+}$-binding property of the aorta and gizzard proteins (K. Takahashi, K. Hiwada, T. Kokubu, unpublished observations, 1987), we named the novel protein calponin (calcium-binding and calmodulin-binding troponin T–like protein).

Materials and Methods

Preparation of Proteins

Chicken gizzard calponin was prepared as described previously. Aorta calponin was purified from fresh bovine aorta according to the method for gizzard calponin with a slight modification: The trimmed smooth muscle from fresh bovine aorta was immediately boiled in a boiling water bath for 2 minutes. The heated muscle was chilled on ice and minced. All subsequent procedures were performed at 4°C. The minced muscle (250 g) was homogenized twice in 1 L of 50 mM imidazole HCl buffer (pH 6.9) containing 300 mM KCl, 5 mM EGTA, 1 mM dithiothreitol (DTT), 0.5
mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM sodium thiosulfate by a Polytron PT 45-80 (Kinematica, Lucerne, Switzerland) at the maximum speed for 30 seconds. After centrifugation at 12,000 g for 20 minutes, the supernatant was centrifuged at 150,000 g for 30 minutes. To 100 ml of filtered supernatant was added 16.4 g of ammonium sulfate and the mixture was stirred for 30 minutes. The resulting precipitate was collected by centrifugation at 12,000 g for 30 minutes. The pellet was dissolved in 25 ml of 20 mM Tris HCl buffer (pH 7.6) containing 6 M urea, 50 mM NaCl, 0.1 mM EGTA, 0.5 mM DTT (Buffer A) and dialyzed against Buffer A for 8 hours, during which the buffer was changed twice (1 L each). The dialyzed sample was centrifuged at 150,000 g for 1 hour, and the supernatant was applied to a SP-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) ion exchange column (1.5 x 30 cm) preequilibrated with Buffer A. The column was eluted with a linear gradient produced by 150 ml of Buffer A and 150 ml of Buffer A containing 300 mM NaCl. The calponin fractions eluted at 60 to 80 mM NaCl were collected, concentrated by ultrafiltration (Model YM-10, Amicon Corporation, Danvers, MA, USA), and gel chromatographed on an Ultrogel AcA 44 column (1 x 5 cm). The column was eluted with 0.1 mM EGTA and 0.5 mM DTT, stored at 0°C, and used within 48 hours.

The following proteins were prepared according to published methods: chicken gizzard and bovine aorta tropomyosin,12 bovine brain calmodulin,13 rabbit skeletal troponin,14 and bovine cardiac troponin T.15 The rabbit skeletal troponin was separated into three components according to the method of Perry and Cole.16 The chymotrypsin digestion of rabbit skeletal and bovine cardiac troponin T, and the purification of T1 and Tα subfragments of rabbit skeletal troponin T were performed as described by Tanokura et al.17 Bovine cardiac troponin T was digested into four major fragments with apparent Mr values of 15,000, 16,000, 27,000 and 31,000 and more than three minor fragments in the Mr range of 11,000 to 13,000.

Preparation of Antibody and Immunoblotting Procedure

Immunoblotting procedure and preparation of the specific rabbit antibody to chicken gizzard calponin were described previously.18 The antibody against bovine cardiac troponin T was raised in rabbits and purified by a previously described method.11 The T1 fragment-specific antibody was prepared by affinity chromatography using T1α-coupled Sepharose 4B column.
was taken to be 70,000. Urea for all chromatographic separations was deionized as a 9 M solution on an Amberlite MB-1 (Rohm and Haas, Woburn, MA, USA) mixed-bed ion exchange resin immediately before use.

Results

Purification of Bovine Aorta Calponin

The result of a typical purification of bovine aorta calponin is shown in Figure 1. Starting from 250 g of minced muscle, about 20 to 30 mg of purified protein was obtained (six different preparations). Its molar ratio relative to tropomyosin in the muscle extract was estimated to be 0.9:1.0 by scanning the stained gels (12.5% acrylamide) based on the molecular weights of calponin ($M_r = 35,000$) and tropomyosin ($M_r = 70,000$). The final preparation showed over 98% pure protein on SDS-polyacrylamide gel electrophoresis (see Figure 1A, Lane 5). As shown in Figure 1C, isoelectric focusing of the purified aorta calponin revealed several isoforms. The major isoelectric variants were detected in the pH region of 8.4 to 9.1.

Molecular Size

When a low dose of aorta calponin was subjected to SDS-polyacrylamide gel electrophoresis, the calponin showed more than four bands in the $M_r$ range of 35,000. The Stokes radii of aorta and gizzard calponins determined by gel chromatography were found to be 27.8 and 27.1 Å, respectively. The $s_{20,w}$ value (sedimentation coefficient at zero concentration, corrected at 20°C in water) of gizzard calponin was estimated to be 3.16. The calculated $M_r$ of gizzard calponin was 35,300 (partial specific volume was assumed to be 0.725 ml/g$^2$).

Amino Acid Composition

Table 1 shows the amino acid composition of aorta and gizzard calponins (mixtures of isoforms). The amino acid compositions of the two proteins were very similar.

Immunological Cross-reactivity with Troponin T

On immunoblotting, as shown in Figure 2B, anti-gizzard calponin antibody specifically stained the $T_2\alpha$ fragment of rabbit skeletal troponin T and 31,000 and 15,000 $M_r$ fragments of bovine cardiac troponin T (Lanes 5 and 6). $T_2\alpha$-specific anti–troponin T antibody recognized the aorta and gizzard calponins (see Figure 2D, Lanes 4 and 5). $T_2\alpha$-specific anti–troponin T antibody cross-reacted with 31,000 and 15,000 $M_r$ fragments of bovine cardiac troponin T as well as $T_2\alpha$ and $T_2\beta\delta$ subfragments of rabbit skeletal troponin T (see Figure 2D, Lanes 2 and 3).

Interaction with Calmodulin

In the absence of Ca$^{2+}$, all of the bound calponin on the calmodulin affinity column was eluted as a single symmetrical peak from the column at 0.1 M NaCl in 20 mM Tris HC1 buffer (pH 7.0) containing 2 mM MgCl$\_2$, 0.5 mM DTT, and 1 mM EGTA in a retarded position of more than 2 column volumes after the change of NaCl concentration. In the presence of 2 mM CaCl$\_2$, the bound calponin was not eluted with the buffer containing 6 M urea. After the column was washed, more than 70% of the bound calponin was eluted at 0.5

**Figure 1.** Purification of bovine aorta calponin and immunoblotting with anti-gizzard calponin antibody (A and B). The same samples stained with Coomassie brilliant blue (A, Lanes 1–5) were applied for the corresponding immunoblot (B, Lanes 1–5). Lane 1, muscle extract; Lane 2, heat-soluble proteins; Lane 3, 30% ammonium sulfate-precipitated proteins; Lane 4, material subjected to the Ultrogel AcA 44 column. Lane 5, purified aorta calponin. Molecular weight markers are indicated on the right side in A: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and $\alpha$-lactalalbumin (14,400). The gel contains 15% acrylamide. Isoelectric focusing of purified aorta calponin (C). The isoelectric point (pl) markers used are trypsinogen (9.30), lentil lectin (8.45), and horse myoglobin (8.30 and 7.30). A = actin; TM = tropomyosin; CN = calponin.
M NaCl in the elution buffer (Figure 3). The remains were not eluted in the presence of 1 M NaCl. When Ca$^{2+}$ was chelated with EGTA, the two calponin fractions were indistinguishable on SDS-polyacrylamide gel electrophoresis (12.5% acrylamide).

**Interaction with Troponin C, Troponin I, and Tropomyosin**

Both aorta and gizzard calponins bound to immobilized troponin C in the presence and absence of Ca$^{2+}$ at less than 0.1 M NaCl in the elution buffer, but they did not bind to troponin I (Table 2). In the absence of Ca$^{2+}$, the aorta and gizzard calponins retained on the troponin C affinity column were eluted at 0.1 M NaCl. In the presence of Ca$^{2+}$, the bound aorta and gizzard calponins were eluted in a retarded position from the column at 0.2 M NaCl. The aorta and gizzard calponins bound to the tropomyosin affinity column. With the application of NaCl gradient from 0 to 0.4 M NaCl, aorta and gizzard calponins were eluted from the corresponding tropomyosin affinity columns as a single symmetrical peak at 0.12 and 0.18 M NaCl, respectively.

**Discussion**

Mechanisms by which Ca$^{2+}$ regulates the contraction in vascular smooth muscle are under heated controversy. With respect to thin filament-linked regulation, it has long been believed that smooth muscle does not contain tropinin or tropomini-like protein. Recent studies have shown that vascular smooth muscle contains thin filament-linked regulatory proteins such as leiotonin and caldesmon.

We purified a novel protein that was antigenically and biochemically related to troponin T from bovine aorta smooth muscle. This protein, which we named calponin, is a calmodulin-binding and tropomyosin-binding protein. The aorta calponin shared a number of common physicochemical characteristics with the gizzard (M$_r$ = 34,000) calmodulin-binding and F-actin-binding protein.

The aorta and gizzard proteins were immunologically cross-reactive. Troponin T has been shown to have a number of isoforms. Willkinson et al. and Breitbart et al. have suggested that isoforms of troponin T may result from differential splicing of a single gene. Calponin also showed several isoforms. The isoforms of calponin may have been generated artifically during the purification procedures or may be products of posttranslational modification.

Our results demonstrated that the aorta and gizzard calponins shared a common antigenic determinant with the COOH-terminal segment of rabbit skeletal and bovine cardiac troponin T. A major isoform of rabbit skeletal troponin T consists of a single polypeptide chain with 259 amino acid residues and can be cleaved into two functionally separate fragments termed T$_1$ (residues 1-158) and T$_2$ (residues 159-259) by treatment with chymotrypsin.

We showed that anti-gizzard calponin antibody did not cross-react with the T$_3$I subfragment (residues 159-242) of rabbit skeletal troponin T. Thus, it seems that the homologous structure between calponin and troponin T exists in the limited COOH-terminal region of the T$_2$ fragment.

Our results showed that the calponin did not interact with troponin I. Troponin I interacts with the T$_2$ fragment at residues 223 to 227 or with a more extended region including the NH$_2$-terminal segment of the T$_2$ fragment. On the other hand, Tanokura et al. have observed that there are two sites for the interaction between troponin C and troponin T: The NH$_2$-terminal region of the T$_2$ fragment is responsible for Ca$^{2+}$-sensitive interaction, and the COOH-terminal region of the T$_2$ fragment is responsible for Ca$^{2+}$-independent interaction. Binding of the aorta and gizzard calponins to troponin C did take place in the presence and absence of Ca$^{2+}$ at less than 0.1 M NaCl in the elution buffer. The binding strength of calponin to troponin C in the presence of Ca$^{2+}$ was much weaker than that of the Ca$^{2+}$-sensitive binding site of the T$_2$ fragment to troponin C, suggesting that the NH$_2$-terminal region of the T$_2$ fragment may not be included in the calponin sequence.

The Ca$^{2+}$ regulatory property of troponin T is mostly localized in the T$_1$ region. In particular, the region around the COOH-terminal residues 243 to 259 is essential for the Ca$^{2+}$-sensitizing activity of troponin T.

**Table 1. Amino Acid Composition of Bovine Aorta Calponin and Comparison with Chicken Gizzard Calponin**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Composition of calponin (mol of residue/100 mol of total residues)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta</td>
</tr>
<tr>
<td>Aspartic acid/asparagine</td>
<td>10.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.1</td>
</tr>
<tr>
<td>Serine</td>
<td>4.7</td>
</tr>
<tr>
<td>Glutamic acid/glutamine</td>
<td>15.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.4</td>
</tr>
<tr>
<td>Valine</td>
<td>4.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.7</td>
</tr>
<tr>
<td>Proline</td>
<td>5.0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.6</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The results represent the average of three different analyses. ND = not determined.

*Extrapolated to zero time hydrolysis.
†72-hour incubation results.

The Ca$^{2+}$ regulatory property of troponin T is most localized in the T$_1$ region. In particular, the region around the COOH-terminal residues 243 to 259 is essential for the Ca$^{2+}$-sensitizing activity of troponin T.
Figure 2. Antigenic relationship between calponin and troponin T. The same samples stained with Coomassie brilliant blue (A and C) were applied for the corresponding immunoblots probed with anti-gizzard calponin antibody (B) and with T2-specific anti-troponin T antibody (D). In A and B: Lane 1, chicken gizzard extract; Lane 2, gizzard calponin; Lane 3, bovine aorta extract; Lane 4, aorta calponin; Lane 5, chymotryptic subfragments of rabbit skeletal troponin T; Lane 6, chymotryptic subfragments of bovine cardiac troponin T. In C and D: Lane 1, bovine cardiac extract; Lane 2, chymotryptic subfragments of rabbit skeletal troponin T; Lane 3, chymotryptic subfragments of bovine cardiac troponin T; Lane 4, gizzard calponin; Lane 5, aorta calponin. The calponins in chicken gizzard and bovine aorta extracts are indicated by arrowheads (Lanes 1 and 3 in A). The same molecular weight markers as in Figure 1 are indicated on the left side in A. The gel contained 15% acrylamide. T1 and T2α are chymotryptic T1 and T2α subfragments of rabbit skeletal troponin T, respectively; bcT is bovine cardiac troponin T.

Table 2. Binding of Aorta and Gizzard Calponins to Immobilized Troponin C, Troponin I, and Tropomyosin

<table>
<thead>
<tr>
<th>Sample applied</th>
<th>+Ca²⁺</th>
<th>-Ca²⁺</th>
<th>TN-C</th>
<th>Aorta TM</th>
<th>Gizzard TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl concentration (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta calponin</td>
<td>0.2†</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1 (0.12)</td>
<td>—</td>
</tr>
<tr>
<td>Gizzard calponin</td>
<td>0.2†</td>
<td>0.1</td>
<td>0.0</td>
<td>—</td>
<td>0.1† (0.18)</td>
</tr>
</tbody>
</table>

Values show the molar concentration of NaCl at which the protein was eluted from the affinity column. Values in parentheses represent the molar concentration of NaCl at each protein peak eluted with NaCl gradient (0.0–0.40 M). The NaCl concentration is taken as a measure of the binding strength of each protein to the ligands.

TN-C = rabbit skeletal troponin C; TN-I = rabbit skeletal troponin I; TM = tropomyosin.

* +Ca²⁺ buffer (pH 7.0) contained 20 mM Tris HCl, 2 mM MgCl₂, 0.5 mM DTT, 2 mM CaCl₂, and various concentrations of NaCl. — Ca²⁺ buffer had the same pH and compositions as the +Ca²⁺ buffer except that 2 mM CaCl₂ was replaced with 1 mM EGTA. The affinity chromatography with troponin I-Sepharose 4B column or tropomyosin-Sepharose 4B column was performed in —Ca²⁺ buffer.

†Samples were eluted in a retarded position of more than 2 column volumes after the change of NaCl concentration in the elution buffer.
Within this and possibly a more extended region (residues 197–250) of the COOH-terminal segment of the T2 fragment, there is a tropomyosin-binding site.30,42 The amino acid sequences in the region of cysteine 190 of skeletal α-tropomyosin, with which the COOH-terminal segment of the T2 fragment interacts, are almost completely conserved in gizzard γ-tropomyosin.43 Considered together with the results of this study, calponin, in association with tropomyosin, may play some regulatory role on smooth muscle thin filament. In support of this possibility, the aorta and gizzard calponins that bound to the corresponding immobilized tropomyosins interacted with a specific region of paracrystals of aorta and gizzard tropomyosin with a 40-nm periodicity (K. Takahashi, K. Hiwada, T. Kokubu, unpublished observations, 1987).

Our preliminary study showed that the immunoreactive form of gizzard calponin was localized on the microfilament bundles (stress fibers) in cultured rat arterial smooth muscle cells.44 Recently, Ngai et al.45 demonstrated that native thin filament prepared from chicken gizzard smooth muscle consisted almost exclusively of actin, tropomyosin, caldesmon, and a 32,000 Mr polypeptide. We have observed that calponin is a major component of the actomyosin preparations from chicken gizzard smooth muscle, and we confirmed that the 32,000 Mr protein (in native thin filament) was identical to the gizzard calponin based on the electrophoretic mobility and the immunological cross-reactivity (K. Takahashi, K. Hiwada, T. Kokubu, unpublished observations, 1987).

In conclusion, a novel troponin T-like protein was purified from bovine aorta smooth muscle. This protein and chicken gizzard 34,000 Mr protein were termed calponin. Although the functional property of the calponin is currently uncertain, its ability to bind to calmodulin and tropomyosin suggests that calponin may be an additional Ca2+ regulatory component of smooth muscle thin filament.

**References**

evidence for three different forms of troponin I in striated muscle. FEBS Lett 1974;40:253–257
K Takahashi, K Hiwada and T Kokubu

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