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Presence of Ca^{2+}-Sensitive and -Insensitive SM22α Isoproteins in Bovine Aorta

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Two proteins (25- and 22-kDa) that cross-react with anti-gizzard SM22α antibody were isolated from bovine aorta. The former, identical to a SM22α homolog reported previously (Kobayashi, R., Kubota, T., and Hidaka, H. (1994) *Biochem. Biophys. Res. Commun.* **198,** 1275-1280), associates with the membrane fraction in the presence of Ca^{2+} and dissociates in the presence of EGTA, while the latter is insensitive to Ca^{2+}. Peptide mapping and partial sequence analysis revealed that the 22-kDa protein is an isoform of the 25-kDa protein, without 22 amino acid residues from the C-terminus. Immunological analysis of the tissue distribution of these proteins showed that the 25-kDa protein exists in lung and spleen besides aorta, while the 22-kDa protein occurs specifically in the aorta and spleen.

It is generally agreed that phosphorylation of myosin light chains plays a major role in the calcium sensitization of smooth muscle tissue (1-3). However, other regulatory systems may also have a role to play in the Ca^{2+}-dependent or -independent regulation of smooth muscle contraction (4,5). Some novel smooth muscle proteins which may regulate the contractile state of muscle, such as calponin, purified from chicken gizzard (6) and bovine aorta (7), which interacts with F-actin, tropomyosin and calmodulin (7), have already been described. In addition, another protein named SM22α has been isolated and characterized from chicken gizzard (8, 9). While searching for a new regulatory factor of vascular smooth muscle, we previously found a Ca^{2+}-sensitive 25-kDa protein in bovine aorta smooth muscle (10). This protein was thought to be a mammalian homolog of chicken gizzard SM22α based on the amino acid sequence.

In the present study, we attempted to verify immunologically the structural homology of the 25-kDa bovine aorta protein and gizzard SM22α. Immunoblot analysis revealed that the 25-kDa aorta protein cross-reacts with anti-gizzard SM22α antibody. In addition, an aorta protein with a slightly lower molecular weight (about 22-kDa) was also found to cross-react with the antibody. It is presumed that the 22-kDa protein is an isoprotein of the 25-kDa aorta protein and also a homolog of the gizzard SM22α. In this study, the 22- and 25-kDa aorta proteins are referred to tentatively as the L- and H-forms (low and high molecular weight forms), respectively. The present report describes the immunological verification of the structural homology of the H-form and gizzard SM22α, as well as the isolation and characterization of the L-form.

MATERIALS AND METHODS

Isolation and purification. Fresh bovine aorta tissue was homogenized by a Polytron homogenizer in 6 vol. of buffer (A) containing Ca^{2+} (20 mM Tris-HCl, 1 mM $CaCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, 100 μg/ml

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Abbreviations: CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; CHAPS, 3-[(3-chloramidepropyl)-dimethylammonio]-1-propanesulfonate; MES, 2-(N-morpholino)ethane-sulfonic acid; PVDF, polyvinylidene difluoride.

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soybean trypsin inhibitor, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin, and 1mM dithiothreitol, pH 7.6) at 4°C. The homogenate was centrifuged at 20,000 g for 20 min. The supernatant was analyzed by one dimensional SDS/PAGE. Extract of aorta with buffer (B) containing EGTA instead of $CaCl_2$ in the buffer A was also analyzed. The pH of aorta extract with the buffer B was adjusted to 4.7 and the mixture was centrifuged at 20,000 g for 20 min. Following readjustment of the pH of the supernatant to 7.0, an ammonium sulfate fraction of 35-70% saturation was collected (20,000 g for 20 min), and re-suspended in 20 mM MES-NaOH buffer solution (pH 6.5). The crude protein was dialyzed overnight against the same solution. The solution was subjected to chromatography on a SP-Sepharose column (2.5 $\times$ 15 cm). The column was developed with 20 mM of MES-NaOH buffer solution (pH 6.5) with a linear gradient of 0-1.0 M NaCl. The L-form was eluted at 0.12-0.16 M NaCl, while the H-form at 0.18-0.21 M NaCl. The fraction containing the L-form was dialyzed against 10 mM of K^+-phosphate buffer (pH 6.8) and subjected to a hydroxylapatite column (1.5 $\times$ 10 cm). The column was developed with 10-250 mM of the phosphate buffer. The L-form was eluted at 55-100 mM phosphate. The H-form was isolated from the its crude protein fraction using methods reported previously (10). Gizzard SM22α protein was isolated from fresh chicken tissue using a previously reported methods (8). Specimens for the analysis of tissue distribution of the L- and H-forms were prepared from bovine brain, lung, heart, aorta, liver, spleen, and kidney. The tissues (2 g) were homogenized separately by a Polytron homogenizer in 5 vol. of 8 M urea at 4°C. Following centrifugation of the homogenates at 20,000 g for 20 min, the supernatant were used for the analysis.

Antibody production. Antibodies to the purified gizzard SM22α and aorta proteins were produced by injecting 1 mg of each protein, emulsified in complete Freund's adjuvant, into rabbits intramuscularly. Booster shots of antigen in the incomplete adjuvant were given twice at 2-week intervals. The rabbits were bled 10 days after the last injection.

Electrophoresis and immunological techniques. One-dimensional SDS/PAGE was performed on 12% gels as described by Laemmli (11) and gels were stained with Coomasie brilliant blue (12). The electrophoretic transfer of proteins from gels to a PVDF-membrane was performed on a semi-dry transfer cell (Bio-Rad, Trans-Blot SD) for 45 min at 8 v using 10 mM CAPS-NaOH buffer (pH 11) in 10%(v/v) methanol as the transfer buffer. For immunodetection of the transferred proteins, the procedure of Burnette (13) was followed except that the second antibody was linked to horseradish peroxidase. Antigen-antibody complexes were visualized by reacting the bound peroxidase with diaminobenzidine and hydrogen peroxide.

Protein sequence analysis. The purified L- and H-forms were digested with lysylendopeptidase according to methods reported previously (14). Proteolytic fragments were separated by reverse-phase HPLC on a C18 column (TOSOH, TSK-GEL ODS-80TM, 4.6 $\times$ 250 mm) using a Shimadzu LC10A HPLC system with a 120-min gradient (0-80% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The amino acid sequence of each fragment was analyzed using an Applied Biosystems 473A protein sequencer.

RESULTS AND DISCUSSION

Identification of Ca^{2+}-sensitive protein and isolation of Ca^{2+}-insensitive protein. To determine Ca^{2+}-dependency of aorta proteins, extracts of tissues with buffer containing Ca^{2+} (buffer A) as well as with buffer containing EGTA (buffer B) were analyzed by one dimensional SDS/PAGE (Fig. 1A). The H-form (25-kDa) was specifically detected in the EGTA-extract but absent in the Ca^{2+}-extract. We have previously reported the primary structure of the H-form (a homolog of chicken gizzard SM22α) based on the amino acid sequence (10). In the present study, the structural homology of the H-form and the SM22α protein was confirmed immunologically. Immunoblot analysis of the aorta extracts indicated that the H-form cross-reacts with anti-gizzard SM22α antibody (Fig. 1B). In addition, an aorta protein with a low molecular weight (about 22-kDa, L-form), present in both the EGTA-extracts and Ca^{2+}-extracts, was also found to cross-react with the antibody (Fig. 1B). It is presumed that the L-form is a homolog of the gizzard SM22α protein and an isoprotein of the H-form.

The H-form is absent in the Ca^{2+}-extract (Fig. 1A), but the addition of the detergent such as CHAPS (0.3%) to the homogenizing buffer enables the extraction of the H-form (Fig. 1C and 1D). Furthermore, in the presence of Ca^{2+}, the H-form was extracted from the precipitate of centrifugation by 5 M guanidine hydrochloride (Fig. 1E and 1F). These facts indicate that the H-form remains associated with some insoluble fraction in the presence of Ca^{2+}. In spite of the structural homology of the H- and L-forms, these isoforms differ in that the presence or absence of Ca^{2+} ion greatly changes the binding activity of the H-form to the membrane or cytoskeleton. The H-form remains associated with the membrane fraction in the presence of Ca^{2+}, but dissociates in the absence of Ca^{2+}, while the L-form is soluble regardless of the

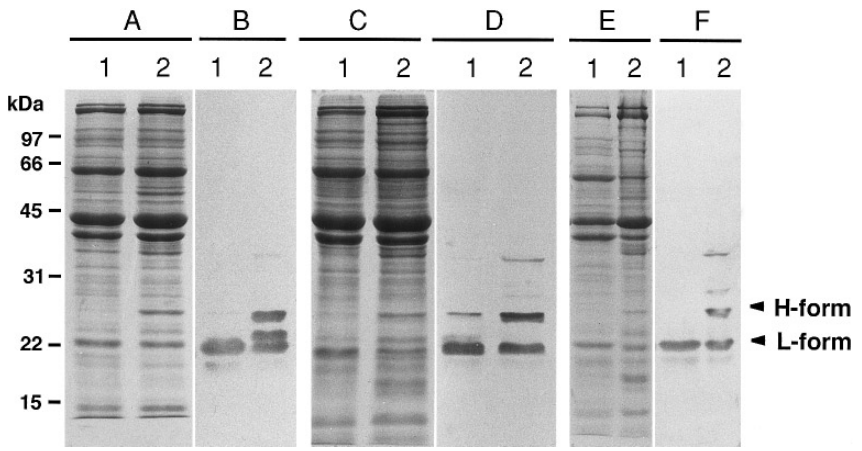

FIG. 1. SDS/PAGE and immunoblot analysis of bovine aorta extracts. A, C, and E: SDS/PAGE stained with coomassie brilliant blue. B, D, and F: Immunoblot proved with rabbit antibody against gizzard SM22 protein and peroxidase-conjugated anti-rabbit IgG. A and B: Lane 1, Ca^{2+}-extract; Lane 2, EGTA-extract. C and D: Lane 1, Ca^{2+}-extract; Lane 2, extract obtained by 20 mM Tris-HCl buffer containing 1mM $CaCl_2$ and 0.3 % CHAPS. E and F: Lane 1, Ca^{2+}-extract; Lane 2, extract of insoluble fraction by 5 M guanidine hydrochloride. Values to the left of the panel show molecular masses.

presence or absence of Ca^{2+}. Thus, the H-form is Ca^{2+}-sensitive, while the L-form is Ca^{2+}-insensitive.

In order to elucidate the structure and character of the Ca^{2+}-insensitive L-form, protein was purified from extract of bovine aorta in essentially the same manner as that used for separation of H-form, i.e. ammonium sulfate fractionation, acid precipitation, and SP-Sepharose and hydroxylapatite chromatography (10). The results of the SDS/PAGE of the purified L- and H-forms are shown in Fig. 4A. Since the L-form was eluted faster than the H-form in the cation-exchange chromatography, the basicity of the L-form was considered to be less than that of the H-form.

Peptide mapping and partial amino acid sequence determination. In order to compare the primary structure of the L- and H-forms, the purified proteins were digested with lysylendopeptidase. The resulting peptides were chromatographed on a C18 reverse-phase column. Peptide mapping of the H- and L-forms showed that the peptides from both proteins were identical excluding two fragments (H and L in Fig. 2A), which were specific to their respective protein. The results of amino acid sequence analyses of the designated peptides (H and L) are shown in Fig 2B. The sequence of fragment H matches residues 153-177 of the aorta H-form reported previously (10), while that of fragment L fits residues 153-158 of the H-form. The results reveal that the L-form is an isoform of the H-form, without 22 amino acid residues from the C-terminal moiety. The difference in the molecular weights of the L- and H-forms (approximately 2290-Da) supports the results of SDS/PAGE. The H-form is considered to be more basic than the L-form, because the 22 amino acid residues of the C-terminal of the H-form contain three arginine residues. This finding matches the basicity of the L- and H-forms observed on the ion-exchange chromatography. As shown in Fig. 1A, the H-form binds the membrane fraction in the presence of Ca^{2+}, while the L-form is soluble regardless of the presence or absence of Ca^{2+}. The difference in the binding activity of these isoforms to the membrane or cytoskeleton may be attributed to the 22 amino acid residues of the C-terminal. It is supposed that a membrane-binding domain, not a Ca^{2+}-binding domain, exists in the C-terminal moiety of the H-form.

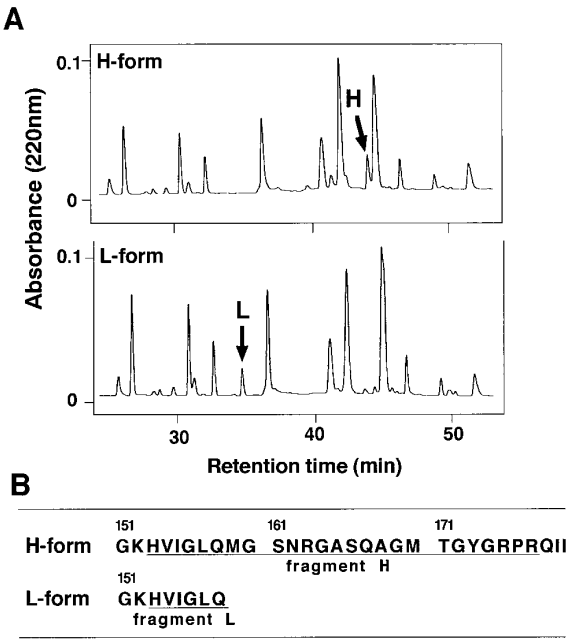

FIG. 2. HPLC and sequence analyses of the H- and L-forms. A, HPLC elution profile of lysylendopeptidase-digested peptides obtained from the H-form (upper panel) and the L-form (lower panel). Peptide fragments designated H and L were sequenced. B, Peptide sequencing data of fragment H of the H-form and fragment L of the L-form. Fragment designations correspond to those shown in A. Residues determined in this experiment are underlined. Superscript numbers indicate the residue numbers for the aorta 25-kDa protein (SM22α homolog) previously reported (10). Details of experimental conditions are described in the text.

It seems unlikely that the L-form is a postmortem degradation product of the H-form, because the L-form was always isolated from fresh bovine aorta tissue regardless of the presence or absence of proteinase inhibitors. In addition, we confirmed by SDS/PAGE and immunoblot analysis that the L-form exists in the aorta of dog which immediately after having killed, as shown in Figures 3A and 3B. These facts support that the L-form exists inherently in bovine and dog aorta. If different processes operate in the splicing of the primary transcript of the aorta H-form gene, two kinds of mRNA may be generated, affording two species of protein. However, results of studies on the structure and expression of a smooth muscle SM22α gene do not support this (15-18). An inherent proteolytic cleavage of the H-form by a specific enzyme, would explain L-form formation.

Cross-reactivity and tissue distribution. In order to examine the immunological cross-reactivity of the L- and H-forms, anti L-form and H-form antibodies were prepared using rabbits. Fig. 4A shows SDS/PAGE of the L- and H-forms. As shown in Fig. 4B and 4C, the anti L-form antibody recognized both the L- and H-form antigens, although the titer was low. On the other hand, the anti H-form antibody reacted only with the H-form antigen but not with the L-form antigen. From these findings, it may be deduced that the antigenic determinant of the H-form is located at the C-terminus, while that of the L-form is located elsewhere.

Tissue distribution of the L- and H-forms was examined immunologically. Fig. 5A shows SDS/PAGE of extracts of bovine brain, lung, heart, aorta, liver, spleen, and kidney. The presence of the protein antigens in these extracts was determined by immunoblot analysis using anti-gizzard SM22 antibody, which recognizes sensitively both the antigens. As shown in Fig. 5B, the H-form exists in lung and spleen besides aorta, and in only trace amounts in

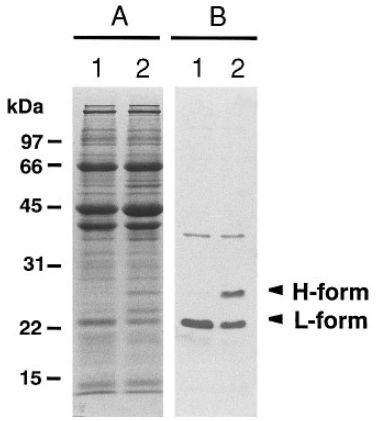

FIG. 3. SDS/PAGE and immunoblot analysis of dog aorta extracts. A, SDS/PAGE analysis of aorta extracts stained with coomassie brilliant blue. Lane 1, Ca^{2+}-extract; Lane 2, EGTA-extract. B, Immunoblot proved with rabbit antibody against gizzard SM22 protein and peroxidase-conjugated anti-rabbit IgG. Lane 1, Ca^{2+}-extract; Lane 2, EGTA-extract. Values to the left of the panel show molecular masses.

liver and kidney, while the L-form occurs specifically in the aorta and spleen. These results seem to imply that the L- and H-forms have a different biological significance. However, Lees-Miller et al. using the anti-chicken gizzard SM22 antibody showed that SM22 is express exclusively in smooth muscle containing tissues in chicken and present in only trace amounts in brain, liver and heart, and that the antibody preparation did not cross-react with extracts of bovine aorta (19). In our present study, the anti-gizzard SM22 antibody cross-reacts with extracts of bovine and dog aorta (Fig. 1B and 3B). Discrepancy of such results might depend on the difference of species and the difference of titer of antibody used.

In summary, we have isolated and partially sequenced a 22-kDa protein (L-form), and determined that the L-form is an isoform of a 25-kDa protein (H-form), a bovine homolog of avian SM22α, without the 22 amino acid residues from the C-terminal moiety. The L-form

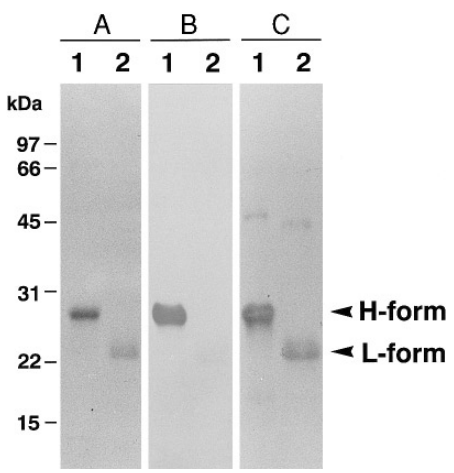

FIG. 4. Immunological cross-reactivity of the L- and H-forms. A, SDS/PAGE stained with coomassie brilliant blue. Lane 1, purified H-form; Lane 2, purified L-form; B, immunoblot proved with rabbit antibody to the H-form. Lane 1, H-form; Lane 2, L-form; C, immunoblot proved with rabbit antibody to the L-form. Lane 1, H-form; Lane 2, L-form.

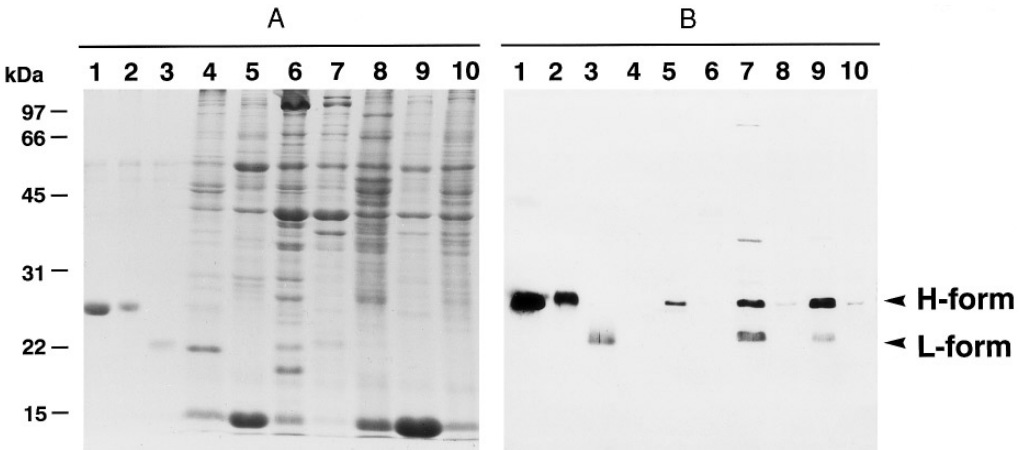

FIG. 5. Immunoblot analysis of the tissue distribution of the H- and L-forms. The homogenates of various bovine tissues were electrophoresed on a 12% (w/v) SDS/PAGE, transferred to a PVDF membrane, and treated with rabbit antibody and with peroxidase-conjugated anti-rabbit IgG. A, stained with coomassie brilliant blue; B, proved with anti-gizzard SM22 antibody. Lanes 1-10: gizzard SM22α, aorta H-form, aorta L-form, brain, lung, heart, aorta, liver, spleen, kidney.

was found to be soluble in the presence or absence of Ca^{2+} and the H-form remains associated with the membrane fraction in the presence of Ca^{2+} but dissociates in the presence of EGTA. Although the inherent functions of these two proteins still remain unknown, the difference in their binding activity to the membrane or cytoskeleton may be attributed to the presence of the C-terminal moiety of the H-form. We are currently exploring the relationship between structure and function in both these isoproteins.

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