Volume 274, number 1,2, 146–150 FEBS 09089 November 1990

Isolation and sequence of a tropomyosin-binding fragment of turkey gizzard calponin

Katia Vancompernolle[1], Mario Gimona[2], Monica Herzog[2], Jozef Van Damme[1], Joël Vandekerckhove[1] and Vic Small[2]

[1] *Laboratory of Physiological Chemistry, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium* and [2] *Institute of Molecular Biology of the Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria*

Received 15 September 1990

Limited chymotryptic cleavage of turkey gizzard calponin yields a 13 kDa fragment which could be purified by its ability to bind to Sepharose-immobilized tropomyosin. This 13 kD polypeptide is shown to be derived from a 22 kDa fragment. Complete amino acid sequence analysis of the 13 kD and 22 kD fragments reveals high homology with the formerly characterized smooth muscle-specific protein SM22α (Pearlstone, J.R., Weber, M., Lees-Miller, J.P., Carpenter, M.R. and Smillie L.B., 1987, J. Biol. Chem. 262, 5985–5991) and the product of gene mp20 of *Drosophila* (Ayme-Southqate, A., Lasko, P., French, C. and Pardue, M.L. [(1989) J. Cell Biol. 108, 521–531]. Furthermore we recognize sequence elements of a putative actin-binding domain of α-actinin, the calpactin I or p 36 sequence, and a consensus motif present in the repeats of the gene product of the candidate *unc*-87 gene of *C. elegans* (S.D. Goetinck and R.H. Waterston, personal communication).

Calponin; Smooth muscle; Tropomyosin binding; Actin-binding site; Sequence

1. INTRODUCTION

The early, pivotal studies on the regulation of contraction of vertebrate smooth muscle pinpointed myosin phosphorylation as the sole Ca^{2+}-dependent switch on the contractile apparatus (see e.g. [1]). Subsequent work has confirmed the primary role of myosin phosphorylation both in vivo as well as in vitro [2] but the discovery of new actin-associated proteins has brought the sole dependence on the myosin switch into question. The identification of two actin- and calmodulin-binding proteins, caldesmon [3,4] and subsequently calponin [5], have suggested that actin-linked regulation may play a modulatory role in smooth muscle. Further, the attractive possibility has been aired that caldesmon, at least, may serve a central function in the maintenance of smooth muscle tone [6].

Calponin, a protein showing heat stable properties, like caldesmon, has been purified from various smooth muscles [7]. This protein, of apparent molecular mass around 34 kDa, has been shown to be immunologically related to skeletal muscle troponin T [8] and capable of binding tropomyosin [9]. More recently, thin filaments have been isolated from smooth muscle tissue that contain calponin [10] and functional tests performed in vitro indicate that calponin inhibits actomyosin ATPase activity [10,11]. We report here amino acid sequence studies on a 13 kDa and a 22 kDa tropomyosin-binding fragment of calponin that reveals homologies to a formerly characterized low molecular weight protein, SM22α from smooth muscle, of unknown function [12,13] and to the product of gene mp20 of *Drosophila*, a protein which is specifically absent from asynchronous oscillatory flight muscle [14].

The calponin fragment also exhibits homology to the actin binding domain of α-actinin [15,16], as well as to a segment of the calpactin I (or p 36) heavy chain [17].

Correspondence address: J. Vandekerckhove, Laboratory for Physiological Chemistry, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

Abbreviations: BNPS-skatole, 2-(2-nitrophenyl-sulphenyl)-3-methyl-3-bromoindolenine; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Protein purification*

Avian gizzard and porcine stomach calponin were purified using a number of modifications of the method given by Takahashi et al. [5]. 500 g of fresh muscle tissue was cleaned, minced and subsequently heated in a microwave oven for 5 min at 700 W. After transfer into a mason jar, ice-cold S-buffer (25 mM Mes, 25 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, pH 5.4) supplemented with 300 mM KCl was poured onto the heated muscle and the suspension homogenized in a Sorvall Omnimixer for 1 min at top speed. Following 30 min extraction, the residue was removed by centrifugation for 10 min at 11 000 rpm in a Sorvall RC-5 using a GSA rotor. The supernatant was filtered through glass wool and brought to 30% ammonium sulphate saturation and stirred for 10 min. The precipitate containing calponin was collected by centrifugation for 20 min at 11 000 rpm, dissolved and dialyzed against S-buffer (overnight, 1 change). The 30% ammonium sulphate supernatant, enriched in caldesmon and tropomyosin, was brought to 65% saturation, the precipitate collected as above and the pellet dissolved in AA-buffer (10 mM imidazol, 30 mM KCl, 1 mM $MgCl_2$, 0.15 mM DTE, pH 7.5) and dialyzed against the same buffer overnight. Low pH dialysis of the calponin fraction

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

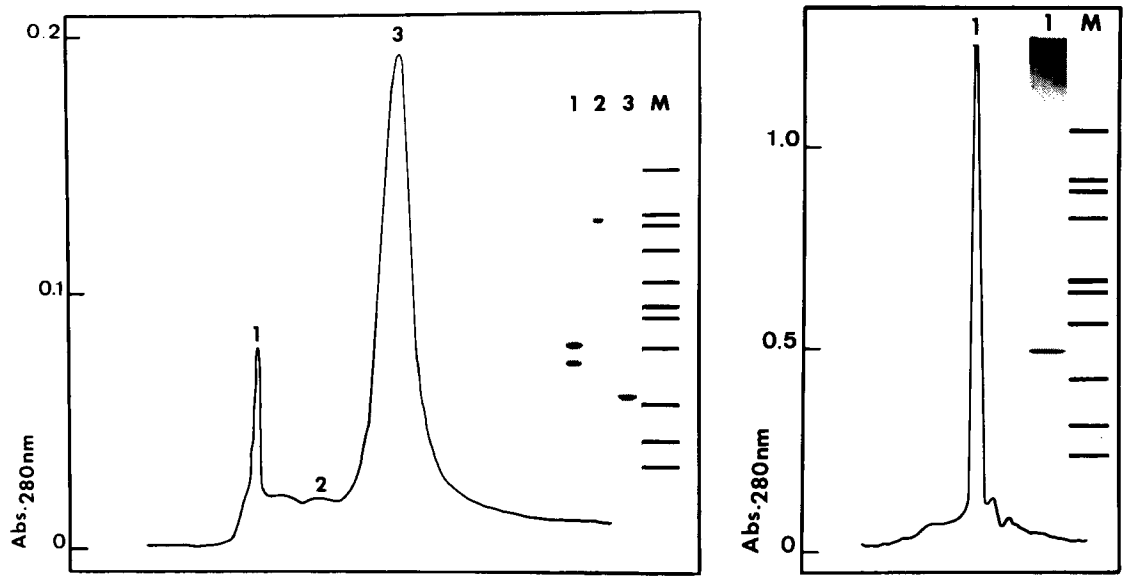

Fig. 1. Purification of turkey gizzard calponin from heat stable extract. (A) Separation on S-Sepharose (gel insets represent peaks for tropomyosin (1), caldesmon (2) and calponin (3). (B) Separation on A-Superose 2 FPLC column (gel inset: purified calponin). M = molecular weight markers. From top to bottom: myosin heavy chain (212), metavinculin (150), vinculin (130), phosphorylase *a* (92.5), transferrin (76), pyruvate kinase (57.2), hexokinase (51), actin (42) carbonic anhydrase (29), myosin light chain (20) and cytochrome *c* (11.7 kDa).

precipitated most of the contaminating tropomyosin, leaving about 60–70% of the calponin soluble. The precipitate was removed by centrifugation in an SS-34 rotor at 18 000 rpm for 20 min and the clarified supernatant applied to a S-Sepharose fast flow column equilibrated in S-buffer. Proteins were eluted with a linear gradient of 25 to 350 mM NaCl. Caldesmon and tropomyosin were purified by DEAE (DE-52) ion exchange chromatography of the dialyzed caldesmon-tropomyosin fraction in AA-buffer using a linear gradient of 30 to 300 mM KCl.

Purified proteins were coupled to CNBr-activated Sepharose (Pharmacia Sweden) according to the manufacturer's instructions.

2.2. *Enzymatic and chemical cleavages*

Limited digestions of calponin with chymotrypsin were performed at a molar ratio of enzyme-to-protein of 1:100 for 0.5–30 min in S-buffer. For electrophoresis, reactions were stopped by adding excess of soybean trypsin inhibitor and then SDS sample mix (with subsequent boiling). For chromatography the digests and rapidly applied to a tropomyosin affinity column of 1 ml volume (Pharmacia HR5/5) attached to an FPLC unit. Calponin fragments were also directly purified by reversed phase HPLC. In this case, the digest was not terminated with inhibitor and immediately loaded onto the column and eluted with a gradient of acetonitrile (see below).

Exhaustive digestions were carried out with the following enzymes: trypsin, endolysine-C and Asp-N protease (all from Boehringer, Mannheim, FRG). The substrate (100 μg) was dissolved and digested with 2 μg of protease in 500 μl 100 mM Tris-HCl buffer, pH 8.5, for 4 h at 37°C, acidified with trifluoroacetic acid (final concentration 1%) and stored at −20°C. The digestion with *Staphylococcus aureus* V8 protease was made in 0.5% NH_4HCO_3 overnight at 37°C, using an enzyme to substrate ratio of 1:50.

Cleavage with BNPS-Skatole was performed according to Fontana [17]. The reaction was terminated by drying in a Speed Vac Concentrator (Savant Instruments, USA). The residue was resuspended in 1 ml water and the reagent extracted with 3×1 ml ethylacetate. The water-layer was retained, made 1% in TFA and saved for HPLC.

2.3. *Protein-chemical methods*

Purification steps, cleavage products and affinity column fractions were monitored by SDS-PAGE using minislab gels cast according to Matsudaira and Burgess [18] and run in the buffer system of Laemmli [19]. Protein electroblotting, combined with NH_2-terminal sequencing was performed as described by Bauw et al. [20]. In situ digestions of polyvinylidene difluoride-bound proteins were carried out as described by Bauw et al. [21]. HPLC peptide separations were done as described previously [22] (for large peptide fragments) or [21] (for the in situ released small fragments). Amino acid sequencing was carried out on an ABI 470 gas-phase sequenator with on-line (model 120A) phenylthiohydantoin amino acid analyser.

3. RESULTS AND DISCUSSION

3.1. *Isolation of calponin and the tropomyosin-binding domains*

Using the described procedure, calponin could be purified to homogeneity without the need of urea denaturation, as employed by Takahashi and colleagues [5]. Fig. 1 illustrates the two column purification steps employed. Affinity columns carrying native calponin prepared in this way, bound both caldesmon and tropomyosin (Fig. 2A,B), with elution of these proteins occurring at around 100 mM KCl. Using affinity columns with either caldesmon or tropomyosin as the bound ligand, calponin was likewise retained (Fig. 2C). These results corroborate the findings of Takahashi et al. [8,9] with respect to the abundancy of calponin in smooth muscle tissue and its binding to tropomyosin. They further add caldesmon to the repertoire of binding partners.

Limited chymotryptic digestion of avian and mammalian calponin gave rise to an identical set of peptides (Fig. 3A) with primary apparent molecular masses of 31–7 kDa (see also [7,8]). After extensive digestion (16–30 min), a rather stable peptide around 22 kDa

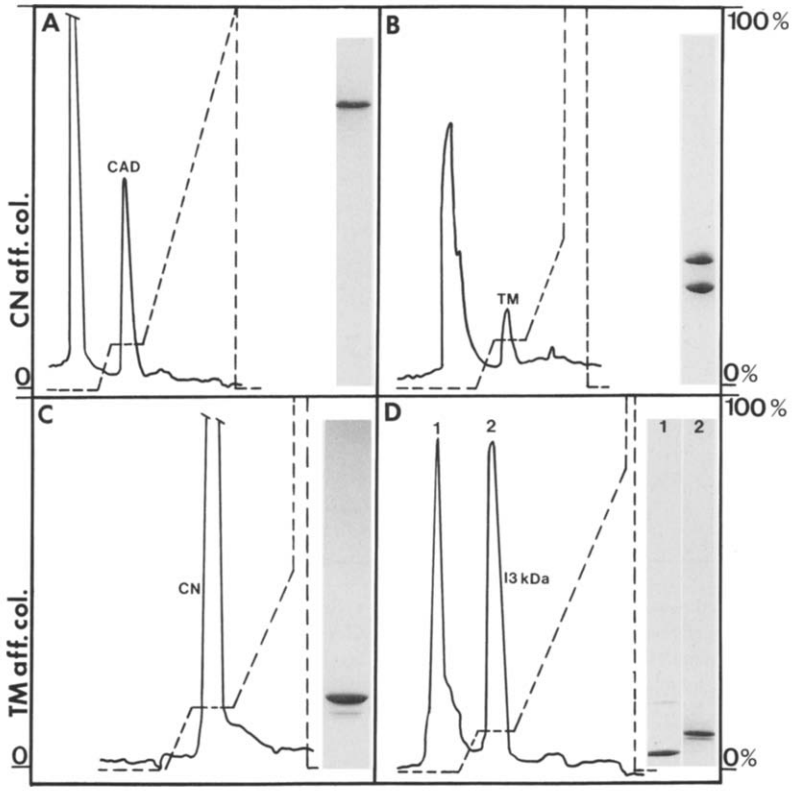

Fig. 2. Binding of calponin to tropomyosin and caldesmon on FPLC affinity columns. Proteins were loaded onto affinity columns and eluted using a programmed linear gradient ranging from 5 (0%) to 1000 mM KCl (100%) and a manual hold at the peak boundaries. Immobilized calponin was able to bind purified caldesmon (A) and tropomyosin (B). The bound proteins eluted at ionic strengths of 70 and 100 mM KCl, respectively. In the reverse assay calponin was bound to a tropomyosin affinity column and eluted likewise at around 100 mM KCl. In (b), a chymotryptic digest corresponding to Fig. 3 (B, 8 min) was applied to the tropomyosin column. Only a 13 kDa peptide was bound and was recovered in much larger amounts than seen in the applied digest (see text).

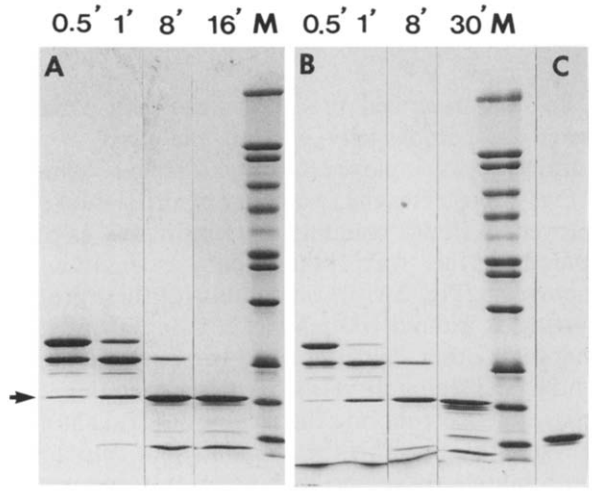

Fig. 3. Chymotrypsin peptide maps of calponin purified from: (A) hog stomach and (B) turkey gizzard. Numbers above gel lanes indicate cleavage times in minutes at room temperature. Identical cleavage patterns were obtained with both proteins and included major peptides of 31, 22 and 7 kDa. Note the high stability of 22 kDa fragment (arrow). (C) the 13 kDa peptide recovered from the tropomyosin affinity column from a digest corresponding to (B), 8 min. M = molecular weight markers as in Fig. 1.

became evident together with a minor product of 13 kDa molecular mass (Fig. 3A,B, 8 min). When such digests were passed over a tropomyosin affinity column the latter 13 kDa peptide was specifically bound (Figs. 2D and 3C) whereas the 22 kDa peptide was, paradoxically, lost on the affinity column, being absent both in the flow-through and the eluted fractions (up to 1 M salt). Correspondingly, the amounts of the 13 kDa piece recovered were noticeably greater than in the applied sample. This result could be explained in terms of the induction of a conformational change in the 22 kDa fragment on interaction with tropomyosin that produces a more open structure in the COOH-terminal segment (Fig. 5), which is more sensitive towards residual chymotryptic activity.

Both the 13 kDa and 22 kDa fragments could be purified directly from the chymotryptic digests by reversed phase HPLC (results not shown). Sequence analysis was performed on both fragments obtained in this latter way as well as on the 13 kDa fragment obtained via the tropomyosin column and then purified by HPLC. The 13 kDa peptides obtained by either route gave identical results.

3.2. *Sequence of the tropomyosin-binding domains*

Unlike calponin, the 13 kDa fragment was not NH_2-terminally blocked and its NH_2-terminal sequence could be determined over 30 residues. The complete amino acid sequence was deduced by aligning the NH_2-terminal sequence with that of overlapping peptides obtained by cleavage with trypsin, endolysin-C, the Asp-N specific protease, *Staphylococcus aureus* V8 protease and BNPS-Skatole (Fig. 4). The 13 kDa fragment consists of 138 amino acids and contains a COOH-terminal tyrosine, in line with the specificity of chymotrypsin used to generate the fragment.

A similar approach was used to elucidate the sequence of the 22 kDa fragment which overlapped that of the 13 kDa fragment and showed a COOH-terminal extension of 38 residues, resulting in a 176 amino acid polypeptide (Fig. 4). The N-terminal sequence of the 22 kDa fragment (which included that of the 13 kDa fragment), was obtained with unusually low initial yields, suggesting that only a fraction was unblocked. Detailed examination of the SDS-PAGE pattern of the 22 kDa fragment (Fig. 3A,B) revealed a doublet consisting of a major 22 kDa and a minor 21 kDa band. Since attempts to separate both components by FPLC or reversed phase HPLC proved unsuccessful the two bands were separated by SDS-PAGE, electroblotted on poly-4-vinyl-*N*-methylpyridine-coated glass-fibre membranes [21] and subjected to NH_2-terminal analysis.

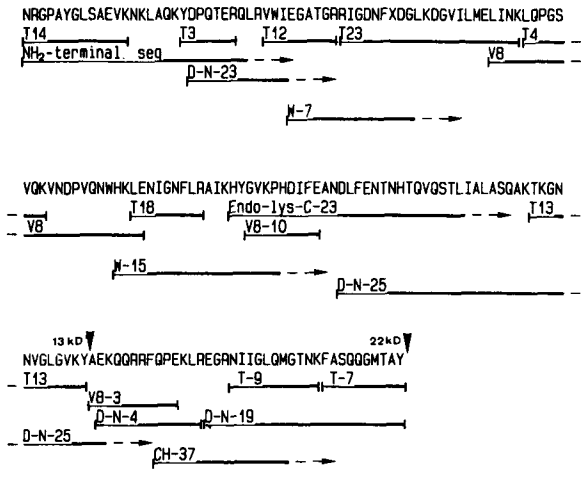

Fig. 4. The complete amino acid sequence and peptide overlaps of the 13 kDa and 22 kDa fragments of turkey gizzard calponin. Only those peptides necessary to document the overlaps are shown. (T) tryptic peptides; (Endo-Lys-C, D-N, V8 CH and W; peptides obtained by cleavage with endolysine C, the aspartic acid N-specific protease, the *Staphylococcus aureus* V8 protease, chymotrypsin and chemical cleavage with BNPS-Skatole, respectively). Peptide numbering refers to their positions in the HPLC chromatograms. Solid bars indicate the sequences spanned by the peptides. Bars terminating with arrows indicate peptides which do not terminate at this position, but were not further sequenced because we had sufficient overlapping sequences from other peptides. Vertical arrows indicate the COOH-terminus of the 13 kDa and 22 kDa fragments, respectively. * Indicates a modified amino acid of unknown nature.

This confirmed indeed that the 22 kDa fragment was blocked and showed that the other sequence corresponded to that of the minor 21 kDa fragment. We conclude that intact calponin and the 22 kDa fragment possess the same blocked N-terminus while the 21 kDa fragment arises by removal of a small segment at this extremity and thus facilitates sequencing access. For convenience we shall continue to refer to the sequenced fragment as the '22 kDa fragment'.

3.3. *Sequence homologies*

The sequence of the 22 kDa fragment showed a remarkable degree of homology with that previously reported for chicken gizzard smooth muscle SM22α [13]. While the overall identity is only 39%, both proteins show a much higher homology at their respective extremities. The 21 NH_2-terminal residues are 57%

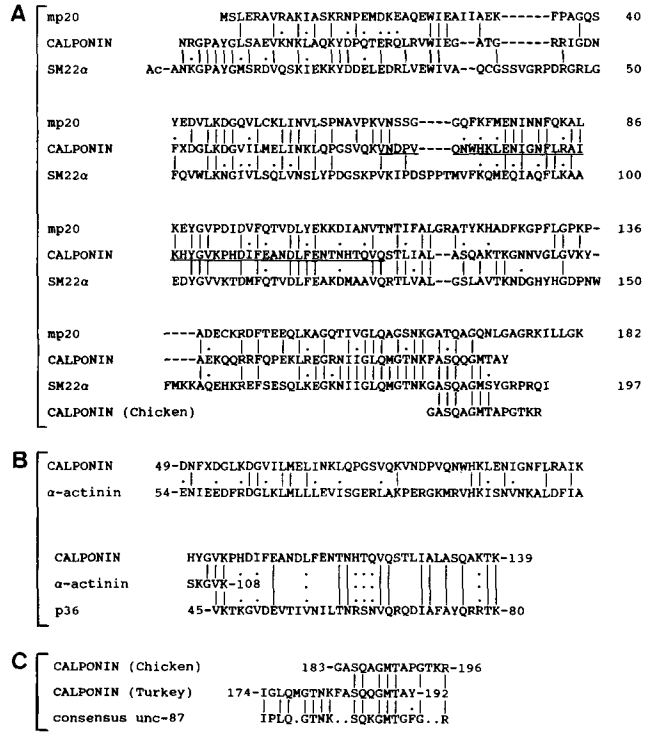

Fig. 5. (A) Homologies between the amino acid sequence of turkey and chicken calponin 22 kDa fragment and the sequences of chicken SM22α and the mp 20 gene product of *D. melanogaster*. (B) Homologies between calponin and sequences observed in chick fibroblast α-actinin and murine p 36. (C) Homologies between chicken and turkey calponin and the consensus sequence of the candidate *unc*-87 gene product of *C. elegans*. Identical and conserved amino acids are connected by bars and dots, respectively. The numbering is made according to that of chick SM22α. Homologous regions in α-actinin and p 36 are flanked by numbers indicating the positions of these segments in their respective proteins. In order to obtain maximal homology between fragment 22 kDa, SM22α and mp 20, we have introduced several gaps in the sequences. The underlined sequence indicates the analysed peptide from chick calponin which was identical to that from turkey calponin. A second chick calponin peptide covering the COOH-terminal part of the 22 kDa fragment, is indicated.

identical, while this score rises to 79% when the 24 COOH-terminal residues are considered. The degree of homology with SM22α was optimized by deleting several segments in the SM22α sequence (residues 39–44, 81–84 and 150–154) (Fig. 5).

In order to exclude the possibility that the observed amino acid differences between chicken SM22α and the 22 kDa calponin fragment of turkey gizzard were simply species-specific differences in corresponding gene products (e.g. SM22α being a proteolytic fragment of the presumed chicken calponin or derived by an alternative splicing mechanism giving rise to either SM22α, calponin and eventually other polypeptides), we also compared partial sequences of chicken and turkey gizzard calponins. These proteins were purified from enriched fractions (see section 2) by SDS-polysaccharide gel electrophoresis, electroblotted on polyvinylidene difluoride membranes and in situ digested with trypsin [21]. The liberated peptides were separated by reversed phase HPLC. Most peptides derived from both proteins were identical, although a few exhibited different retention times (results not shown). This suggested that chicken and turkey calponins were similar but non-identical proteins. Two chicken calponin peptides were subjected to sequence analysis and the results compared with sequences of the 22 kDa fragment of turkey gizzard calponin. The first covered an internal segment and was identical to the corresponding turkey calponin sequence. The second peptide corresponded to the COOH-terminal part of the 22 kDa domain (Fig. 5) and was slightly different from that of SM22α. These data demonstrate minor differences between chicken and turkey calponins, but more importantly that SM22α and chicken calponin must be derived from different structural genes.

The 22 kDa fragment is also 37% identical to the product of gene mp20 of *D. melanogaster* (Fig. 5). This protein is present in every muscle tissue except for the asynchronous oscillatory flight muscles. This particular expression pattern has been connected with the contraction frequency difference observed between asynchronous and synchronous muscle in this organism. On a similar way it may be possible that calponin plays a role in the regulation of the smooth muscle tone.

Segments from the middle part of the 22 kDa fragment show also a weak but significant homology (28% identical and 16% similar residues over a length of 54) to a part of the presumed F-actin binding domain of α-actinin that is itself homologous to the corresponding domain of *Dictyostelium* gelation factor and dystrophin [14,15,23–25]. The adjacent middle segment of the 22 kDa piece is similarly homologous (37% identical and 20% similar amino acids over a length of 35) to the NH_2-terminal Geisow fragment of p36 [16]. As both α-actinin and p36 are known to interact with F-actin, the identified calponin segments could represent parts of single or multiple F-actin binding domains. If so, F-actin and tropomyosin-binding sites could be located close enough to each other to interact cooperatively. A similar organisation of F-actin and tropomyosin-binding sites was also reported for caldesmon [26,27].

Acknowledgements: We thank Mrs C. Schöppl and K. Persyn for typing and Miss K. Koppelstatter and Mrs M. Klappacher for photography. This work was supported in part by a grant from the Austrian Science Research Council and from the Belgian National Fund for Scientific Research. K.V. is bursar of the Belgian Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw.

REFERENCES

[1] Small, J.V. and Sobieszek, A. (1980) Int. Rev. Cytol. 64, 241–306.
[2] Kamm, K.E. and Stull, J.T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620.
[3] Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) Proc. Natl. Acad. Sci. USA 78, 5652–5655.
[4] Sobue, K., Kanda, K., Toshihiko, T. and Ueki, N. (1988) J. Cell. Biochem. 37, 317–325.
[5] Takahashi, K., Hiwada, K. and Kokubu, T. (1986) Biochem. Biophys. Res. Commun. 141, 20–26.
[6] Marston, S.B. (1989) J. Muscle Res. Cell Motil. 10, 97–100.
[7] Takahashi, K., Hiwada, K. and Kokubu, T. (1987) Life Sci. 41, 291–296.
[8] Takahashi, K., Hiwada, K. and Kokubu, T. (1988) Hypertension 11, 620–626.
[9] Takahashi, K., Abe, M., Hiwada, K. and Kokubu, T. (1988) J. Hypertension, 6 Suppl., s40–s43.
[10] Winder, S.J. and Walsch, M.P. (1990) J. Biol. Chem. 265, 10148–10155.
[11] Nishida, W., Abe, M., Takahashi, T. and Hiwada, K. (1990) FEBS Lett. 268, 165–168.
[12] Lees-Miller, J., Heeley, D.H., Smillie, L.B. and Kay, C.M. (1987) J. Biol. Chem. 262, 2988–2993.
[13] Pearlstone, J.R., Weber, M., Lees-Miller, J.P., Carpenter, M.R. and Smillie, L.B. (1987) J. Biol. Chem. 262, 5985–5991.
[14] Arimura, C., Suzuki, T., Yanagisawa, M., Imamura, M., Yamada, Y. and Masaki, T. (1988) Eur. J. Biochem. 177, 649–655.
[15] Baron, M.D., Davison, M.D., Jones, P., Patel, B. and Critchley, D.R. (1987) J. Biol. Chem. 262, 2558–2561.
[16] Saris, C.J.M., Tack, B.F., Kristensen, T., Glenney, J.R. and Hunter, T. (1986) Cell 46, 201–212.
[17] Fontana, A. (1972) Methods Enzymol. 25, 419–423.
[18] Matsudaira, P. and Burgess, D.R. (1978) Anal. Biochem. 87, 386–396.
[19] Laemmli, U.K. (1970) Nature 99, 680–685.
[20] Bauw, G., De Loose, M., Inzé, D., Van Montagu, M. and Vandekerckhove, J. (1987) Proc. Natl. Acad. Sci. USA 84, 4806–4810.
[21] Bauw, J., Van Damme, J., Puype, M., Vandekerckhove, J., Gesser, B., Ratz, G.P., Lauridsen, J.B. and Celis, J.E. (1989) Proc. Natl. Acad. Sci. USA 86, 7701–7705.
[22] Gimona, M., Small, V., Moeremans, M., Van Damme, J., Puype, M. and Vandekerckhove, J. (1988) EMBO J. 7, 2329–2334.
[23] Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) Cell 53, 219–228.
[24] Noegel, A., Witke, W. and Schleicher, M. (1987) FEBS Lett. 223, 391–396.
[25] Blanchard, A., Chanian, V. and Critchley, D.R. (1989) J. Muscle Res. Cell Motil. 10, 280–289.
[26] Fujii, T., Ozawa, J., Ogoma, Y. and Kondo, Y. (1988) J. Biochem. 104, 734–737.
[27] Katayama, E., Horiuchi, K. and Chacko, S. (1989) Biochem. Biophys. Res. Commun. 160, 1316–1322.