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Molecular Cloning and Characterization of Human Non-Smooth Muscle Calponin[1]

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cDNA clones encoding a calponin isoform with 309 amino acids have been isolated from human heart. The deduced amino acid polypeptide (M_r 33,697) showed a neutral isoelectric point of 7.1. The mRNA, expressed in cultured smooth muscle cells as well as in fibroblasts, vascular endothelial cells, and keratinocytes, contains a 3′ untranslated region of 1.2 kilobases that includes an *Alu* repetitive sequence in the antisense direction. On the basis of the nucleotide sequence identity to an expressed sequence tag, HUM21ES93 [Cheng, J.-F., Boyartchuk, V., and Zhu, Y. (1994) *Genomics* 23, 75-84], the human neutral calponin gene is assigned to chromosome 21q11.1. The amino acid sequence indicates that this protein is the human equivalent of mouse calponin-h2 (94.8% identity) [Strasser, P., Gimona, M., Moessler, H., Herzog, M., and Small, J.V. (1993) *FEBS Lett.* 330, 13-18]. Three tandem repeats of 29 amino acids, a Vav-homologous region and an actin-binding sequence, originally identified in the basic calponin isoform, are conserved. There are two consensus phosphorylation sites for tyrosine kinase. An immunoreactive form of the neutral calponin appears to be localized with vinculin in the cell-to-cell junctions of cardiomyocytes. Mouse calponin-h2 is also expressed in both embryonic and adult heart. These results indicate that the human neutral calponin is a non-smooth muscle isoform, and may play a physiological role in cytoskeletal organization.

Key words: actin-binding protein, *Alu* sequence, calponin, cardiac muscle, Vav proto-oncogene.

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Calponin was originally isolated as an actin-associated basic protein from chicken gizzard and bovine aortic smooth muscles (SMC) (*1, 2*). The binding of calponin to the actin filament inhibits actin-activated myosin MgATPase activity *in vitro* (*3-16*), and decreases the shortening velocity of smooth muscle fibers without affecting myosin phosphorylation (*17-19*). Although these studies suggested that calponin regulates the contraction of smooth muscle, much evidence shows the presence and localization of immunoreactive forms of calponin in non-muscle cells (*20-23*) as well as in the cytoskeletal domain of smooth muscle cells (*24-26*). Another line of evidence has revealed the molecular diversity of mammalian calponins. Two isoforms, called calponin-h1 and -h2, encoded by different genes have been cloned from mouse uterus and porcine stomach smooth muscles (*27*). Judging from the sequences of the mouse and porcine cDNA clones, the calponin-h1 isoform is the equivalent of the previously identified basic calponin (*28*), which is predominantly expressed in smooth muscle cells (*29-34*). More recently, cDNA clones encoding a novel acidic calponin isoform with a deduced 330-amino-acid polypeptide (M_r 36,377) have been isolated, and shown to be expressed in both smooth muscle and non-muscle tissues of adult rat (*35, 36*). The acidic isoform is therefore a good candidate for the immunoreactive form of non-muscle calponin.

During the course of studying calponin expression in coronary arteries of human heart, we found that a polyclonal antibody raised against the basic isoform of aortic calponin strongly stains the intercalated disc of cardiomyocytes. Based on the results of molecular cloning and sequence analysis of cDNAs from a human heart library, we show that the immunoreactive form of calponin expressed by cardiomyocytes is a distinct calponin isoform with a neutral isoelectric point. The encoded protein exhibits extensive homology to the mouse calponin-h2 isoform, which was shown to be expressed in both embryonic and adult heart. We also demonstrate that the human neutral calponin is expressed by cultured smooth muscle cells as well as by non-muscle cells, including fibroblasts, vascular endothelial cells and keratinocytes.

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; NP 40, Nonidet P40; DTT, dithiothreitol.

EXPERIMENTAL PROCEDURES

Antibodies—A polyclonal antibody against the basic isoform of bovine aortic calponin was generated in rabbits and purified by antigen-affinity chromatography according to the method described in Ref. *20*. Anti-calponin monoclonal (clone hCP) and anti-vinculin monoclonal (VIN-11-5) antibodies were purchased from Sigma (St. Louis, USA) and BioMakor (Rehovot, Israel), respectively. The specificities of the antibodies were verified on immunoblotting of total homogenates of human aortic smooth muscles.

Immunofluorescence Microscopy—Human heart ventricle tissue was obtained from autopsied patients. The specimens were rinsed with PBS and mounted in O.C.T. compound (Miles, Elkhart, USA), and then frozen using liquid nitrogen. The frozen sections were cut with a cryostat (5 μm thick), mounted on glass slides, air dried, and then fixed in 100% acetone at -20°C for 7 min. After being rinsed in PBS for 15 min, the sections were incubated with 1% (v/v) goat serum/PBS for 1 h at room temperature. After washing in PBS, the sections were incubated with the primary antibody in 2% (w/v) BSA/PBS overnight at 4°C. They were then washed 7 times with 0.005% (v/v) Tween 20/PBS, followed by incubation with the rhodamine-conjugated goat anti-rabbit or anti-mouse IgG (TAGO Immunologicals, Camarillo, USA) in 2% (w/v) BSA/PBS for 1 h at room temperature. After being washed in 0.005% (v/v) Tween 20/PBS, they were examined under a fluorescence microscope (Olympus Vanox-S).

Immunoelectron Microscopy—Canine cardiac muscle specimens were fixed with 100 mM sodium-phosphate buffer (pH 7.4) containing 0.1% (v/v) glutaraldehyde and 3% (w/v) paraformaldehyde at 4°C for 1 h. The specimens were dehydrated with graded concentrations of methanol and then embedded in Lowicryl K4M resin according to the method of Armbruster *et al.* (*37*). Immunocytochemical staining of the ultrathin sections was performed according to the method of Roth *et al.* (*38*). Anti-caponin polyclonal and anti-vinculin monoclonal antibodies were used as the primary antibodies. Immunogold labeling was carried out using conjugates of 5 and 10 nm colloidal gold with goat anti-rabbit and goat anti-mouse antibodies, respectively (Amersham International, UK). Sections were examined under a JEOL 100 CX electron microscope operated at 80 kV.

SDS-PAGE and Immunoblotting Procedure—Human heart ventricle tissue from an autopsied patient was stored at -30°C. The tissue was homogenized at 4°C in 8 volumes of a RIPA lysis buffer comprising 150 mM NaCl, 1% (v/v) NP 40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris-HCl (pH 8.0), PMSF, and leupeptin. The homogenate was clarified by ultracentrifugation at 100,000 $\times g$ at 4°C for 1 h.

To solubilize components of Z bands and intercalated discs (*39*), the heart tissue, cut into 1-2 mm cubes, was extracted with 2 volumes of a urea solution comprising 3 M urea, 1 mM DTT, 1 mM EGTA, PMSF, and leupeptin at 4°C for 3 h with gentle shaking. After centrifugation at $20,000\times g$ for 10 min, the supernatants were analyzed by SDS-PAGE. An equal amount of protein was loaded on each lane, as judged on densitometoric analysis (Ultroscan XL Enhanced Laser Densitometer, Pharmacia LKB) of the Coomassie Brilliant Blue-stained gels. The transfer and immunoblotting procedures were carried out as previously described (*20, 40*).

cDNA Cloning and Sequencing—A λ ZAPII human heart cDNA library (Stratagene, La Jolla, USA) constructed from an adult Caucasian left ventricle was screened with a full length cDNA probe (1,536 bp) of human basic calponin, which was labeled with digoxigenin (DIG) using the DIG-High Prime labeling mixture (Boehringer Mannheim, Germany). The nucleotide sequence of the full length human basic calponin cDNA isolated from aortic smooth muscle has been deposited in the DDBJ/GenBank™/EMBL Data Bank (accession No. D17408). Prehybridization and hybridization were performed in a solution of 250 mM sodium-phosphate buffer (pH 7.2) containing 20% (w/v) SDS, 1 mM EDTA, and 0.5% (w/v) blocking reagent (Boehringer Mannheim) at 50°C.

Screening of 5.6×10^5 plaques of the human adult heart library resulted in the isolation of two distinct classes of clones. A representative of each class of clones was isolated, subcloned into the pBluescript SK($-$) vector (Stratagene), and then sequenced. One class of clones contained a nucleotide sequence identical to that of basic calponin (nt No. 30 to 1480), and the other contained a 807-bp sequence (nt No. 214 to 1020 of human neutral calponin) which shows a high degree of homology to the sequence of mouse calponin-h2 (*27*). The immunoreactivity of IPTG-induced β-galactosidase fusion proteins encoded by each class of λ phage clones was examined using a picoBlue Immunoscreening Kit (Stratagene). Using the 807-bp cDNA fragment as a probe, we further screened 8.8×10^5 plaques of another cDNA library constructed in the λgt11 vector (Clontech, Palo Alto, USA) prepared from a female Caucasian whole heart. The prehybridization and hybridization were performed in the same solution as for the first screening at 60°C. Screening of the latter library resulted in the identification of 12 clones containing the neutral calponin sequence, which on the basis of the insert size fell into three categories (1,790, 2,060, and 2,130 bp in length). DNA sequencing was carried out on both strands using an Applied BioSystem model 373A DNA sequencer according to the manufacturer's instructions.

All restriction and DNA modifying enzymes were purchased from New England Biolabs (Beverly, USA) and Boehringer Mannheim.

Cell Culture—Primary cultured cells were purchased from Kurabo Biomedical Business (Osaka); NHDF (normal human skin fibroblasts, HF4001), NHEK (normal human epidermal keratinocytes derived from neonatal foreskin, HK4001), HUVEC (human umbilical vein endothelial cells, HE4101), and aortic VSMC (human aortic smooth muscle cells, HS4001). The cells were cultured in the recommended medium at 37°C under a humidified atmosphere containing 5% (v/v) CO_2. The second passage cells were used for experiments.

RNA Preparation—Total RNA was extracted from cultured cells (60-70% confluency) according to the method described by Stallcup and Washington (*41*). Total RNA from cardiac muscle of BALB/c mice was extracted using an ISOGEN RNA extraction kit (Nippon Gene, Toyama).

Reverse Transcription-PCR Analysis—Reverse transcription (RT) of 0.5 μg of total RNA was carried out using a reaction mixture of a first-strand cDNA synthesis kit

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(Pharmacia Biotech, Uppsala, Sweden). After 60 min incubation at 37°C, 25 pmol of each of the primers and 2.5 U of *Taq* DNA polymerase (Pharmacia Biotech) were added, and then the total volume was adjusted to 50 μl with water. Combinations of forward (5′-CTG CAG AGC GGG GTG GAC ATT GGC-3′ for human neutral calponin nt No. 433 to 456, 5′-AGC CCC GCC GGT CCC GCT GG-3′ for mouse calponin-h2 nt No. 53 to 72, and 5′-GTC TGT GTC ATC TGC ACC TC-3′ for mouse calponin-h1 nt No. 52 to 71) and reverse (5′-GCC GGC CTC CTC CTG GTA GTA AGG-3′ for human neutral calponin nt No. 951 to 928, 5′-CAA ACT GAT GTG AAA GAG AT-3′ for mouse calponin-h2 nt No. 1365 to 1346, and 5′-TCC CGT CGC AGG AAT GGG GC-3′ for mouse calponin-h1 nt No. 1346 to 1327) primers were selected for amplification, with 30 cycles of denaturation (94°C, 40 s), annealing (57°C, 30 s), and polymerization (72°C, 1 min 30 s).

PCR of Human Genomic DNA—Genomic DNA was extracted from leukocytes of a healthy volunteer using a Stratagene DNA extraction kit (Stratagene). One microgram of DNA was double-digested with *Hinc*II and *Apa*I, and then used as the template for the PCR reaction. Combinations of forward (5′-ACA GAA TTC AGA CCC TCA GAC C-3′, nt No. 1507 to 1528) and reverse (5′-AGG GCT GGG GAC TCC TTA AAA C-3′, nt No. 1962 to 1941) primers were selected to amplify the 3′ untranslated region (residues 1507 to 1962) of the genomic sequence, with 35 cycles of denaturation (94°C, 40 s), annealing (55°C, 30 s), and polymerization (72°C, 1 min 30 s). Cytosine at nt position 1512 was replaced with adenine (underlined) in order to make an *Eco*RI site for subcloning and sequencing.

RESULTS

Expression of a Distinct Calponin Isoform at Cell-to-Cell Junctions of Cardiomyocytes—Immunoreactive forms of calponin were detected in intercalated discs, cell-to-cell junctions of cardiomyocytes, by a polyclonal antibody raised against the basic calponin purified from bovine aorta (Fig. 1a, arrowheads). The immunoreactive calponin expressed by smooth muscle cells of small arteries was also labeled with this antibody (Fig. 1a). However, a monoclonal antibody, clone hCP, raised against human uterine calponin exclusively stained vascular smooth muscle cells (Fig. 1b).

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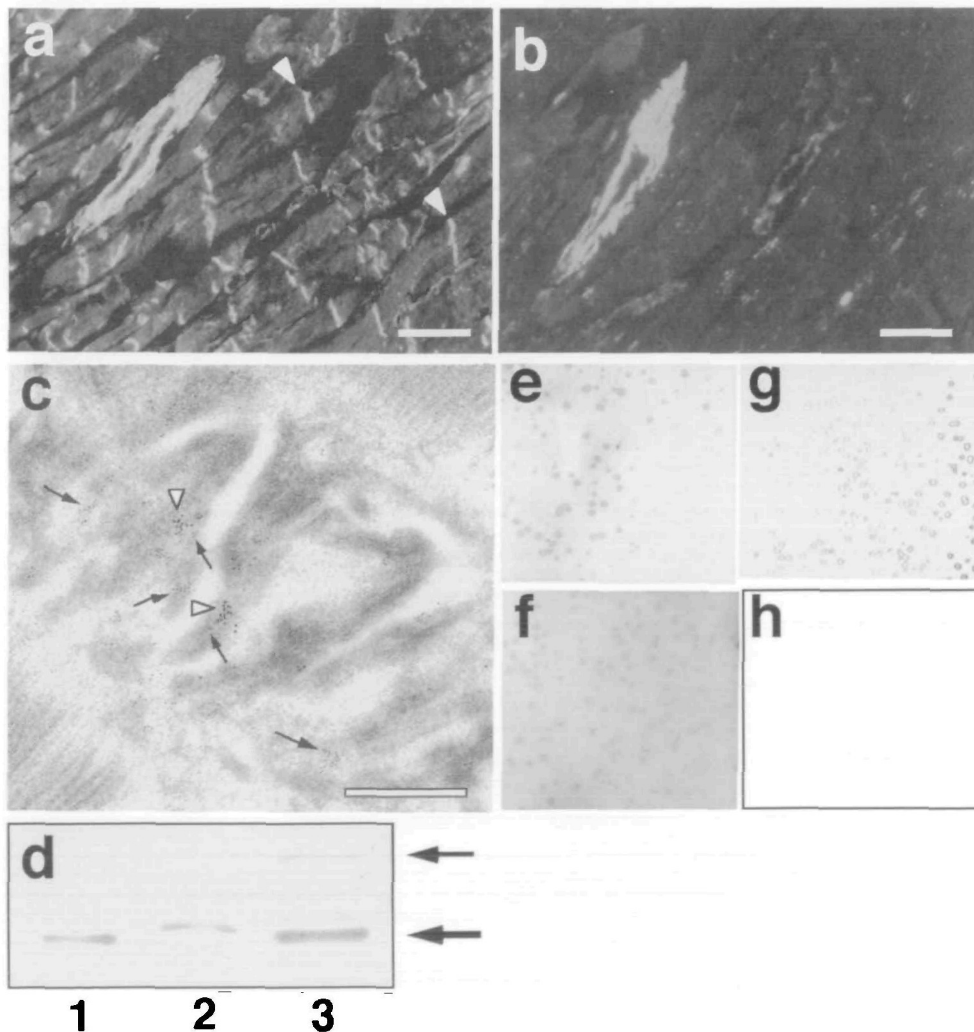

Fig. 1. **Calponin expression in the heart.** a and b: Immunofluorescence microscopy of the human heart. Serial sections of frozen human cardiac tissues were labeled with a polyclonal antibody to bovine aortic calponin (a) or with a monoclonal antibody, clone hCP, to human uterine calponin (b). The arrowheads indicate immunofluorescence-staining of the intercalated discs. The scale bar represents 10 μm. c: Immunoelectron microscopy of the cell-to-cell junctions of a dog cardiomyocyte probed with the polyclonal antibody to calponin (arrows, 5-nm gold particles) and a monoclonal antibody to vinculin, clone VIN-11-5 (open arrowheads, 10-nm particles). The scale bar represents 100 nm. (d): Immunoblots of an SDS-PAGE gel probed with the polyclonal antibody to calponin. Lane 1, human coronary artery extracted with RIPA lysis buffer; lane 2, human whole cardiac muscle extracted with RIPA lysis buffer; lane 3, intercalated disk-, and Z-band-rich fraction of human cardiac muscle extracted with a 3M urea solution (*39*) (see "EXPERIMENTAL PROCEDURES"). The arrow indicates the position of a major 36-37 kDa polypeptide. The thin arrow indicates an additional minor polypeptide with a molecular mass of 40-42 kDa. e, f, g, and h: Immunoblots of IPTG-induced β-galactosidase fusion proteins probed with the polyclonal antibody to calponin (e and f) or the monoclonal antibody, clone hCP (g and h). The β-galactosidase fusion proteins were generated from cDNA clones encoding the basic calponin isoform (nt. No. 30 to 1480) (e and g) or the neutral calponin isoform (nt. No. 214 to 1020) (f and h).

a

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                   GCCCGTCCCG CCGCCCGCCC GCCAGCC ATG AGC TCC ACG CAG TTC AAC AAG GGC CCC TCG   60
                                                 Met Ser Ser Thr Gln Phe Asn Lys Gly Pro Ser   11

TAC GGG CTG TCG GCC GAG GTC AAG AAC CGG CTC CTG TCC AAA TAT GAC CCC CAG AAG GAG  120
Tyr Gly Leu Ser Ala Glu Val Lys Asn Arg Leu Leu Ser Lys Tyr Asp Pro Gln Lys Glu   31

GCA GAG CTC CGC ACC TGG ATC GAG GGA CTC ACC GGC CTC TCC ATC GGC CCC GAC TTC CAG  180
Ala Glu Leu Arg Thr Trp Ile Glu Gly Leu Thr Gly Leu Ser Ile Gly Pro Asp Phe Gln   51

AAG GGC CTG AAG GAT GGA ACT ATC TTA TGC ACA CTC ATG AAC AAG CTA CAG CCG GGC TCC  240
Lys Gly Leu Lys Asp Gly Thr Ile Leu Cys Thr Leu Met Asn Lys Leu Gln Pro Gly Ser   71

GTC CCC AAG ATC AAC CGC TCC ATG CAG AAC TGG CAC CAG CTA GAA AAC CTG TCC AAC TTC  300
Val Pro Lys Ile Asn Arg Ser Met Gln Asn Trp His Gln Leu Glu Asn Leu Ser Asn Phe   91

ATC AAG GCC ATG GTC AGC TAC GGC ATG AAC CCT GTG GAC CTG TTC GAG GCC AAC GAC CTG  360
Ile Lys Ala Met Val Ser Tyr Gly Met Asn Pro Val Asp Leu Phe Glu Ala Asn Asp Leu  111

TTT GAG AGT GGG AAC ATG ACG CAG GTG CAG GTG TCT CTT CTC GCC CTG GCG GGG AAG GCC  420
Phe Glu Ser Gly Asn Met Thr Gln Val Gln Val Ser Leu Leu Ala Leu Ala Gly Lys Ala  131

AAG ACT AAG GGG CTG CAG AGC GGG GTG GAC ATT GGC GTC AAG TAC TCG GAG AAG CAG GAG  480
Lys Thr Lys Gly Leu Gln Ser Gly Val Asp Ile Gly Val Lys Tyr Ser Glu Lys Gln Glu  151

CGG AAT TTC GAC GAT GCC ACC ATG AAG GCT GGC CAG TGC GTC ATC GGG CTG CAG ATG GGC  540
Arg Asn Phe Asp Asp Ala Thr Met Lys Ala Gly Gln Cys Val Ile Gly Leu Gln Met Gly  171

ACC AAC AAA TGC GCC AGC CAG TCG GGC ATG ACT GCC TAC GGC ACG AGA AGG CAT CTC TAT  600
Thr Asn Lys Cys Ala Ser Gln Ser Gly Met Thr Ala Tyr Gly Thr Arg Arg His Leu Tyr  191

GAC CCC AAG AAC CAT ATC CTG CCC CCC ATG GAC CAC TCG ACC ATC AGC CTC CAG ATG GGC  660
Asp Pro Lys Asn His Ile Leu Pro Pro Met Asp His Ser Thr Ile Ser Leu Gln Met Gly  211

ACG AAC AAG TGC GCC AGC CAG GTG GGC ATG ACG GCT CCC GGG ACC CGG CGG CAC ATC TAT  720
Thr Asn Lys Cys Ala Ser Gln Val Gly Met Thr Ala Pro Gly Thr Arg Arg His Ile Tyr  231

GAT ACC AAG CTG GGA ACC GAC AAG TGT GAC AAC TCC TCC ATG TCC CTG CAG ATG GGC TAC  780
Asp Thr Lys Leu Gly Thr Asp Lys Cys Asp Asn Ser Ser Met Ser Leu Gln Met Gly Tyr  251

ACG CAG GGC GCC AAC CAG AGC GGC CAG GTC TTC GGC CTG GGC CGG CAG ATA TAT GAC CCC  840
Thr Gln Gly Ala Asn Gln Ser Gly Gln Val Phe Gly Leu Gly Arg Gln Ile Tyr Asp Pro  271

AAG TAC TGC CCG CAA GGC ACA GTG GCC GAT GGG GCT CCC TCG GGC ACC GGC GAC TGC CCG  900
Lys Tyr Cys Pro Gln Gly Thr Val Ala Asp Gly Ala Pro Ser Gly Thr Gly Asp Cys Pro  291

GAC CCG GGG GAG GTC CCT GAA TAT CCC CCT TAC TAC CAG GAG GAG GCC GGC TAC TGA GGC  960
Asp Pro Gly Glu Val Pro Glu Tyr Pro Pro Tyr Tyr Gln Glu Glu Ala Gly Tyr ***      309

TCCCAGCACG CTCTCTCCCC ACATCGTCTT CCCATCTGGG TTTTTGGGTT TTTCTGTGTT TTCATCTTTT 1030
TTTTTTTTTT TCTTGACCCG TTCAGTGCTG CCAGTCAACC AAGGGTCTGT GAGTGTCAGC GTGGGATCAG 1100
GCAGCAGAGC TTTTTTCCCC TTTGCCTTGA TCCTTCGCAA GGCTGAGCCA CTGGGCTGTG GGGGAAGGGG 1170
TCAAGGCCAT ATCCCAATAC GTGTAGGGCG AGGGTCCCTG CTGGCACATT CAGGCTGTGC TGGGAAGAAG 1240
AGACCTGGGC TTGGAAGGAA CCGGTCCCCG ACGGTTTCTG GTTGCCTCGC CTCTTCCCCC TTTTGTCAGC 1310
TGAGCAGTTT GTGGTTTCTA TGCCCGCAAG TTTCAGGAAG TATTCACAAA AGAAAAATAC ATTTTTTCCC 1380
CCAGGGGTGG GGCAAGGACA GTGGAGAGAG TGCTAGGAAA TGAGTCCCCT GGGAAAGGGG ACCGGGCCGT 1450
GATGTTAAAT ATCTCCGGCT CCCAAGTGAC TGGATTTGCC TAGGACCTTC AGATCAACAG ACTTCAGACC 1520
CTCAGACCTG CCCCGGGGCC AGGTGGAGAA AGTGAGGGCC GTACAAGGAA GTGAAATTCT GAGTTGTTGG 1590
GGCTAAGCCT GACCCCCTCT CCATGCTCCC CGCCCCAACT CACTCTGGCC TCAGTAGATT TTTTTTTCAG 1660
TTGTGGTTGT TGCCCAGGCT GGAGTGCAGT GGCGCCATCT TGGCTCACTG CACCTCCACC TTCCGGGCTC 1730
AAGCGATTCT CCAGCCTCAG CCTCCTGAGT AGCTAGGACT GCAGGTGCTC CACCACGCCC GGCTAATTTT 1800
TGTATTTTTA GTAGAGATGG GGTTTCCCCA TGTTGGCCAG GCTGGTCTCG AACTCCTGGC CTCAGGTGTG 1870
ATCCGCCCGC CTCCGCCTCC CCAAGCGCTG AGATTACAGG TGTGAGCCAC CGTGCCCAGG CCCTCAGTAG 1940
GTTTTAAGGA GTCCCCAGCC CTCCTCCCTT CTGGGCCCGA CCAGCTTATA CTGCTCCATC TTCCCCGGCC 2010
ACATGCCCCG CCAAGTACTG CACAGGGACC CCCCACCCAG GGGCCCTGCT CCGTGAGATA ATGTGAAATA 2080
CGACTGTGGA CCAAACGGAA TAAACCTTT GTTTGTAGGA AGAAAAAAAA                       2130
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b

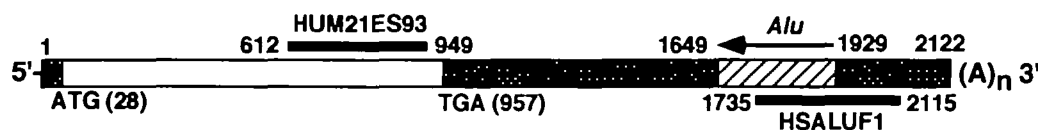

Fig. 2. **Nucleotide and predicted amino acid sequences of the cloned cDNA.** (a) Nucleotide sequence of the full-length insert of human neutral calponin isolated from cardiac muscle. The underlined sequence is homologous to the complementary sequence of Alu consensus (*46*). A polyadenylation signal (AATAAA) at residues 2098-2104 is boxed. (b) Schematic representation of the cloned calponin cDNA and two partial cDNA clones HUM21ES93 (*44*) and HSALUF1 (*45*), in relation to the corresponding nucleotide sequences of the human neutral calponin cDNA.

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These results indicate that an immunoreactive protein (or proteins) expressed by cardiomyocytes is a molecular entity distinct from the smooth muscle-specific isoform of calponin. Double-label immunoelectron microscopy confirmed that the calponin label is concentrated at the inner surface of the cell-to-cell junctions of cardiomyocytes with no cross-reactivity with the sarcomere (Fig. 1c). Some of the label for calponin (Fig. 1c, arrows) is clearly co-localized with that for viculin (Fig. 1c, arrowheads) at the fascia adherens of the intercalated disc membranes, where the actin microfilaments terminate (*42*). Expression of the immunoreactive calponin at the cell-to-cell junctions was detected in mouse and rat cardiomyocytes with this polyclonal antibody (data not shown). As shown in Fig. 1d, the molecular mass of the cardiac muscle calponin detected by the polyclonal antibody on an SDS/polyacrylamide gel is 36-37 kDa (lane 2), which is slightly higher than that of the 36 kDa smooth muscle calponin (lane 1). The 36-37 kDa polypeptide is a major immunoreactive component in the intercalated disc and Z-band (lane 3), and is not recognized by the monoclonal antibody, clone hCP (data not shown). An additional immunoreactive protein with a molecular mass of 40-42 kDa was extracted as a minor component (lane 3).

We isolated two distinct classes of clones encoding calponin isoforms from λZAPII human cardiac muscle

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MSSTQFNKGPSYGLSAEVKNRLLSKYDPQKEAELRTWIEGLTGLSIGPDF   50 H.neutral(h2)
__________________________________S_______________   50 P.h2
__________________________________S_______________   50 M.h2
___AH__R__A__________K_AQ___H_R_Q___E_____V__RR__NN   50 H.basic(h1)
___AH__R__A__________K_AQ___H_Q_Q___E_____V__RR__NN   50 P.basic(h1)
___AH__R__A__________K_AQ___H_R_Q___E_____V__RR__NN   50 M.basic(h1)
__M_H________________KIA____H_A_ED__N____EV__M____N   48 H.acidic
__M_H________________KIA____Q_A_ED__N____EV__MG___TN  48 R.adidic

QKGLKDGTILCTLMNKLQPGSVPKINRSMQNWHQLENLSNFIKAMVSYGM  100 H.neutral(h2)
_______I__________________________________________  100 P.h2
_______V__________________________________________  100 M.h2
MD_____I___EFI_______K___E_T_________IG____ITK___V  100 H.basic(h1)
MD_____I___EFI_______K_V_E_T_________IG____ITK___V  100 P.basic(h1)
MD_____I___EFI_______K_V_E_T_________IG____ITK___V  100 M.basic(h1)
_L_____I___E_I_______K_V_E_SL__P_____IG____IQA____   98 H.acidic
_L_____I___E_I_______K_V_E_SL__P_____IG____IQA____   98 R.acidic

NPVDLFEANDLFESGNMTQVQVSLLALAGKAKTKGLQSGVDIGVKYSEKQ  150 H.neutral(h2)
_______________________________________D__________  150 P.h2
__________________________________________________  150 M.h2
K_H_I_________NT_H_____ST_____SM______..NK_NV___A__  148 H.basic(h1)
K_H_I_________NT_H_____ST_____SM______..NK_NV___A__  148 P.basic(h1)
K_H_I_________NT_H_____ST_____SM______..NK_NV___A__  148 M.basic(h1)
K_H_I_________N________TT_V_____L_____FHTTI_____A__  148 H.adidic
K_H_I_________N________TT_V_____L_____FHTTI_____A__  148 R.acidic

ERNFDDATMKAGQCVIGLQMGTNKCASQSGMTAYGTRRHLYDPKNHILPP  200 H.neutral(h2)
Q_________________________________________________  200 P.h2
__________________________________________________  200 M.h2
__K_EPGKLRE_RNI_______F___Q_______________LGTDQ__  198 H.basic(h1)
__K_EPEKLRE_RNI_______F___Q_______________LGTDQ__  198 P.basic(h1)
__R_EPEKLRE_RNI_______F___Q_______________LGTDQ__  198 M.basic(h1)
T_R__EGKL_____S___________A_______________MQTDK__  198 H.acidic
T_R__EGKL_____S___________A_______________MQTDK__  198 R.acidic

MDHSTISLQMGTNKCASQVGMTAPGTRRHIYDTKLGTDKCDNSSMSLQMG  250 H.neutral(h2)
__________________________________________________  250 P.h2
__C_______________________________________________  250 M.h2
L_QA_________G___A________K_Q_FEPG__MEH___TLNV____  248 H.basic(h1)
L_QA_________G___A________K_Q_FEPG__MEH___TLNV____  248 P.basic(h1)
L_QA_________G___A________K_Q_FEPG__MEH___TLNV____  248 M.basic(h1)
F_QT_________G___A__L_______D___Q_TLQPV_____TI____  248 H.acidic
F_QT_________G___A__S_______D___Q_TLQPV_____TI____  248 R.acidic

YTQGANQSG:QVFGLGRQIYDPKYC..PQGTVADGAPSGTGDCPDPGEVPE  298 H.neutral(h2)
___________________________..___PA____AAA_____G____S(*) 296 P.h2
___________________________..___SA_____A_D_Q...._____A  294 M.h2
SNK__S_R_MT_Y___P__V_______..LTPEYPELGEPAHNHHAHNYYNSA  297 H.basic(h1)
SNK__S_R_MT_Y___P__V_______..LTPEYPELGEPAHNHHPHNYYNSA  297 P.basic(h1)
SNK__S_R_MT_Y___P__V_______..LNPEYPELSEPTHNHHPHNYYNSA  297 M.basic(h1)
TNKV_S_K_MS_Y_______V______AA_TEP_IHNGSQ____TNGSEISDSD  299 H.acidic
TNKV_S_K_MS_Y_______V______AA_TEP_IHNGSQ____TNGSEISDSD  299 R.acidic

YPPYYQEEAGY                                          309 H.neutral(h2)
_LA_C                                                305 M.h2
_QAE_PD_YHGEYQDDYPRDYQYSDQGIDY                       329 H.acidic
_QAE_PD_YHGEYPDEYPREYQYGDDQGIDY                      330 R.acidic
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Fig. 3. **Amino acid sequence similarities to basic, h2, and acidic calponin isoforms.** Sequence alignment between the human neutral calponin and previously identified h2, basic (h1) and acidic calponin isoforms. Only non-identical residues are shown. Residues boxed by a dotted line comprise the actin binding domain (*14*). The shadowing indicates the characteristic tandem repeats. The asterisk (*) at the end of pig calponin-h2 indicates the missing C-terminus (*27*). Other abbreviations: P. basic (h1) and P. h2; pig calponins, basic (h1) and h2 (*27*). M. basic (h1) and M. h2; mouse calponins, basic (h1) and h2 (*27*). R. acidic; rat acidic calponin (*35*). H. basic and H. acidic; human calponins, basic (*1, 68, 69*) (DDBJ/GenBank™/EMBL Data Bank accession No. D17408) and acidic (*56*).

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cDNA libraries. One class of clones contained a nucleotide sequence identical to that of the basic calponin cDNA isolated from human aortic smooth muscles (DDBJ/GenBank™/EMBL Data Bank accession No. D17408), and the other contained a novel nucleotide sequence which shows a high degree of homology to that of mouse calponin-h2 (*27*). The polyclonal antibody cross-reacted with the β-galactosidase fusion proteins encoded by both the two classes of λ phage clones (Fig. 1, e and f). However, the monoclonal antibody, clone hCP, which is monospecific to the smooth muscle calponin, only recognized the basic calponin protein (Fig. 1, g and h). These results suggest that the calponin-like protein (or proteins) expressed by cardiomyocytes may be derived from a cDNA clone homologous to mouse calponin-h2.

Molecular Cloning of Human Neutral Calponin—A full length clone for cardiac muscle calponin was isolated by screening of a λgt11 human whole heart cDNA library with a 807-base pair cDNA clone containing a nucleotide sequence homologous to that of mouse calponin-h2. Twelve cDNAs were isolated, representing at least 3 distinct classes of overlapping clones on the basis of the insert size. There was no isoform variant generated through alternative mRNA splicing. In the longest cDNA isolated, containing a 2,130-base-pair sequence, a single open reading frame (ORF) was found (Fig. 2a). Starting at the first in-frame ATG, 27-base pairs from its 5′ end, the ORF sequence encodes a 309-amino-acid peptide with a predicted molecular mass of 33,697 and an isoelectric point of 7.1 (net charge +2.5 at neutral pH). The in-frame nonsense codon (TGA) is followed by 1173 nt of a 3′ untranslated sequence. A polyadenylation signal is present 18 bp upstream of the poly(A) tail. The full length nucleotide sequence was used to carry out a FASTA search (*43*) of the DDBJ/GenBank™/EMBL databases. The searches revealed that the sequence of HUM21ES93 (GenBank™ accession No. L25193) on human chromosome 21q11.1 (*44*) is identical to the nucleotide sequence, 612 to 949, of the cloned cDNA (Fig. 2b). Furthermore, the complementary sequence of clone HSALUF1 (GenBank™ accession No. X05126) isolated from human fibroblasts as an Alu sequence-containing cDNA (*45*) is identical to nucleotide sequence 1735 to 2115 of the cDNA (Fig. 2b). The nucleotide sequences of clones T97947 and R50270 from the WashU-Merck EST Project, and HSA34C041 (GenBank™ accession No. Z13216) from Genexpress also showed identity to the regions spanning nucleotide sequences 46 to 335, 1508 to 1848, and 783 to 1095, respectively (data not shown). The striking feature of the 3′ untranslated region is the presence of a repetitive sequence of the human Alu family (*46*) in the anti-sense direction (nucleotides 1649 to 1929 of the cDNA) (Fig. 2, a and b). The complementary *Alu* sequence in the cloned cDNA has all of the structural features of the consensus *Alu* sequence (*46*) (86.3% identity). It possesses left and right monomeric units connected by an adenine-rich linker and an additional adenine-rich sequence at its 3′ end. In order to verify that the *Alu* sequence is present in all copies of the mRNA, we attempted PCR amplification of the reverse transcribed mRNA from human heart and aortic VSMC as well as genomic DNA from Japanese male ($n=2$) and female ($n=4$) volunteers, using primers flanking the *Alu* sequence (see "EXPERIMENTAL PROCEDURES"). Analysis of the amplified 456 bp sequence revealed that the *Alu* sequence in the anti-sense direction was unequivocally present in all the mRNA and genomic sequences examined (data not shown).

The deduced amino acid sequence revealed that the neutral calponin shows a high degree of homology to the mouse calponin-h2 isoform (*27*) (94.8% identity) (Fig. 3). The predicted protein contains three 29-amino acid direct repeats (amino acids 166 to 194, 206 to 234, and 245 to 272), which was originally identified in the basic calponin sequence (*1, 28*). Comparison of the amino acid sequence with those of the basic and acidic calponins indicates that the sequence spanning the first repeating motif (amino acids 166 to 194) shows a high degree of homology to the corresponding region of the human basic and acidic calponin isoforms (93% identity to the basic calponin and 96% to the acidic calponin). In contrast, the sequence of the carboxyl-terminal region (amino acids 230 to 309) including the third repeating motif shows substantial diversity among the three classes of calponin, indicating an isoform-specific function of this domain. Of note is that there are two consensus phosphorylation sites for tyrosine kinase in the carboxyl-terminal domain (Tyr^{299} and Tyr^{309}), corresponding tyrosine residues being also present in the mouse calponin-h2 sequence. Although the amino-terminal segment of the actin-binding sequence (*14*) (amino acids 145 to 163 of the chicken (*28*) and human basic calponins, see Fig. 3), Ala^{145} to Glu^{153}, is conserved as the sequence, Ser^{147} to Asp^{155}, 4 nonconservative amino acid substitutions are located on its carboxyl-terminal segment, Asp^{156} to Val^{165}. The phosphorylation sites for protein kinase C (*16, 47, 48*), Ser^{177} and Thr^{186}, are conserved. The predicted phospholipid binding domain (*49*), [Leu^{85} to Ile^{95} of the chicken basic calponin (*28*)], is homologous to the sequence, Leu^{84} to Met^{94}.

The sequence of the amino-terminal region of the cloned

```
                                                          ▼
human vav                32  ELAQAIRDGVILCQLINNLLPHAINLREVNLRIQMSQF.LCLKNIRTFLST  81
human neutral calponin   50  .FQKGLKDGTILCTLMNKLQP.GS.VPKIN...RSMQNWHQLENLSNFIKA  94
human basic calponin     49  NFMDGLKDGIILCEFINKLQP.GS.VKKIN...ESTQNWHQLENIGNFIKA  94
human acidic calponin    47  NFMLGLKDGIILCELINKLQP.GS.VKKVN...ESSLNWPQLENIGNFIKA  92
yeast CDC24                   47  FQQGAPLCILFNSVKPQ....FKLPVIAS.DDLKVCKKSIYDFILG  87

human vav                82  CCEKFCLKRSELFEAFDLFDVQDFGKVIYTLSALS  116
human neutral calponin   95  MV.SYGMNPVDLFEANDLFS.GNMTQVQVSLLALA  127
human basic calponin     95  IT.KYGMKPHDIFEANDLFENTNHTQVQSTLLALA  128
human acidic calponin    93  IQ.AYGMKPHDIFEANDLFENGNMTQVQTTLVALA  126
yeast CDC24              88  CKKHFAFNDEELFTISDVFANSTS.QLVKVLEVVE  121
```

Fig. 4. **Amino acid sequence similarities to the human Vav protooncogene product and yeast CDC24.** Sequence alignment between the human Vav protooncogene product, human calponin isoforms and yeast CDC24. Identical residues are enclosed within black boxes. Periods (.) are introduced for optimal alignment. The arrowhead indicates the breakpoint for oncogenic transformation of Vav (*50, 51*).

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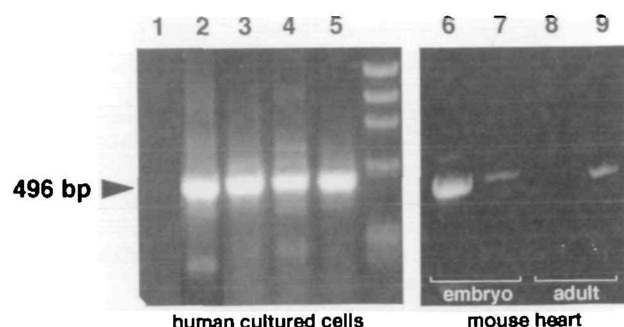

Fig. 5. **Detection of human neutral calponin and mouse calponin-h2 transcripts by reverse transcription PCR.** Left panel, PCR-amplified products from cDNAs generated in reverse transcriptase reactions from 0.5 μg of total RNA purified from cultured human fibroblasts (lane 2), aortic VSMC (passage 4) (lane 3), umbilical vein endothelial cells (lane 4), and epidermal keratinocytes (lane 5). Lane 1, negative control PCR amplification in which the template RNA was omitted, but the reverse transcriptase enzyme was included. The sense and anti-sense primers for human neutral calponin are described under "EXPERIMENTAL PROCEDURES." DNA fragments with the predicted mobility of 496 base pairs are indicated by the arrowhead. Right panel, PCR-amplified products from cDNAs generated in reverse transcriptase reactions from 0.5 μg of total RNA from mouse embryonic (E13.5) (lanes 6 and 7) and adult (lanes 8 and 9) hearts, using sense and anti-sense primers specific for mouse calponin-h1 (lanes 6 and 8) or calponin-h2 (lanes 7 and 9) sequences.

neutral calponin shows extensive homology to the amino-terminal domain of the *vav* protooncogene product (*50, 51*) (Fig. 4). Within 79 residues of the human Vav sequence (residues 37 to 115), there are 39% identity and 62% similarity to the human calponin isoforms. Furthermore, as noted by Adams *et al.* (*52*), this region is similar to the sequence of CDC24 of budding yeast, an essential polarity-determination gene required for the organization of the actin cytoskeleton at the bud site (*53*).

Expression of Human Neutral Calponin in Smooth Muscle and Non-Muscle Cells—To examine the expression of transcripts encoding neutral calponin in cultured human cells, including aortic VSMC, skin fibroblasts, epidermal keratinocytes and umbilical vein endothelial cells, cDNAs were generated by reverse transcription and then amplified by PCR. As shown in Fig. 5, a major PCR product from cDNAs with the predicted mobility of 496 base pairs was generated from RNA isolated from both smooth muscle and non-smooth muscle cells.

Mouse Calponin-h2 Is Expressed in Both Embryonic and Adult Heart—Structural characterization of the neutral calponin indicated that it is the human equivalent of mouse calponin-h2. We therefore examined whether or not transcripts of the mouse calponin-h2 gene are also expressed in the heart. To determine the temporal patterns of gene expression, RT-PCR experiments were performed on the total RNA of hearts prepared from staged mouse embryos and adult mice using primers specific for murine calponin-h2 cDNA (see "EXPERIMENTAL PROCEDURES"). We also studied the expression of the basic calponin isoform in the heart using primers specific for murine calponin-h1 cDNA. Both calponin-h2 and -h1 transcripts of 1,294 and 1,276 base pairs in length, respectively, were detected in the developing heart tube as early as in postcoital day (E) 9.5 mouse embryos (data not shown). In E13.5 embryos, expression of the calponin-h1 mRNA in the heart was greater than that of calponin-h2 mRNA (Fig. 5, lanes 6 and 7). In adult mouse heart, however, the calponin-h2 mRNA was predominant over the calponin-h1 mRNA (Fig. 5, lanes 8 and 9). This pattern of expression of the murine calponin-h1 gene in the developing heart has been confirmed recently by *in situ* hybridization analysis (*32, 34*).

DISCUSSION

In this study, we report an isoform of human calponin with a neutral isoelectric point (pI 7.1) from cardiac muscle. The predicted amino acid sequence demonstrates that this protein is the human equivalent of mouse calponin-h2. Expression of the cDNA clone not only in passaged cultured aortic VSMC but also in cardiac and non-muscle cells indicates that the distinct neutral calponin isoform described here is a non-smooth muscle isoform. Its cellular localization to the cell-to-cell junctions of cardiomyocytes suggests its potential involvement in the organization of the actin cytoskeleton and/or regulation of the cell-to-cell adhesion mechanism.

Structural analysis of the cDNAs encoding calponin isoforms has revealed the presence of three classes of genes with distinct expressional regulation (*27, 28, 35*). Taking the results of these and our studies together, each of the three calponin genes encodes a distinct class of isoforms categorized into acidic (pI 5-6), neutral (pI 7-8), and basic (pI 8-10) calponins, on the basis of the isoelectric point. The basic calponin gene (also called calponin-h1) encodes the originally isolated (*54, 55*), and major calponin isoform which is predominantly smooth muscle-specific (*28-34*). In addition, α (high molecular)- and β (low molecular)-isoforms encoded by the basic calponin gene have been found as products of alternative mRNA splicing (*28, 33*). The neutral calponin described here is the equivalent of calponin-h2, isolated from mouse uterus and porcine stomach smooth muscle tissues (*27*). The functional significance of the expression of a distinct calponin-h2 form in adult smooth muscle tissues remains to be elucidated (*27*). In the present study, however, we have shown that a human equivalent of the mouse calponin-h2 gene is expressed by non-muscle cells as well as by smooth muscle cells. Notably, an expressed sequence tag HUM21ES93 in the chromosome region, 21q11.1 (*44*), which contains a partial cDNA sequence, 612 to 949, of our human calponin clone (Fig. 2b), is expressed in fetal brain tissue. This supports the conclusion that the neutral (h2) isoform of calponin is not specific to smooth muscle cells. The acidic calponin, another class of gene encoding a non-muscle isoform, is also expressed in heart and brain tissues as well as in cultured smooth muscle cells (*35, 36, 56*). However, quantitative PCR amplification of a human cardiac muscle cDNA library, using specific primers which can discriminate between acidic and neutral calponin cDNAs, suggested that expression of the neutral isoform is approximately 4 times greater in the human heart tissue (M.T., H.M., and K.T., unpublished observation). We therefore infer that most of the immunoreactive forms of calponin in cardiomyocytes correspond to the neutral calponin isoform represented by the cDNA described here. Future immunolocalization studies involving isoform-specific antibodies are needed to determine whether or not each isoform is co-expressed in a

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single cell, and if so, it is also necessary to examine whether or not each isoform is localized in a distinct intracellular domain of the actin cytoskeleton.

We found an *Alu* repetitive sequence in the anti-sense direction in the 3' untranslated sequence of the cDNA. The *Alu* family of repetitive elements constitutes about 5% of the total mass of the human genomic DNA. They are frequently transcriptionally silent and are removed by splicing during mRNA processing (*57*). However, some *Alu* sequences are represented by cytoplasmic poly(A)$^+$ mRNA in a spatiotemporally regulated manner (*58-60*). A cDNA clone, HSALUF1, containing the partial nucleotide sequence, 1735 to 2115, of the human neutral calponin, in which the anti-sense *Alu* sequence is included (see Fig. 2b), was used for Northern blot analysis of poly(A)-containing RNAs from normal human tissues and tumors (*45*). Small *Alu*-containig poly(A)$^+$ RNAs were identified on hybridization with this probe in various tissues examined. This raises the intriguing possibility that they might act as anti-sense RNAs for the 3' untranslated region of the human neutral calponin mRNA, which could influence its stability, translational efficiency, and intracellular localization.

A fundamental question concerns the physiological role of the neutral calponin in smooth muscle as well as in non-smooth muscle cells. Its specific localization at the cell-to-cell junctions of cardiomyocytes and co-distribution with vinculin strongly suggests that it may function in the structural organization of the actin filaments and/or their anchorage, and may also be involved in the cell adhesion mechanism. Furthermore, we recently reported that an immunoreactive form of the neutral calponin is apparently co-localized with cadherin on the cytoplasmic surface of the cell-to-cell junctions of keratinocytes (*61*). Hence the neutral calponin may well be physically associated with molecules localized in these structures. These observations are consistent with the reported distribution of calponin in smooth muscle cells in the cytoskeletal actin domain and in the cytoplasmic dense bodies as well as in the adhesion plaques on the cell surface (*26*). It is noteworthy that consensus phosphorylation sites for tyrosine kinase were identified in the carboxyl-terminal sequence of human neutral calponin as well as in that of mouse calponin-h2, and phosphotyrosine residues are concentrated at the cell-to-cell and cell-to-matrix adherens junctions, including the intercalated discs of cardiomyocytes (*62*). It should be noted that there are characteristic tandem repeats of the consensus sequence for tyrosine phosphorylation in the carboxyl-terminal domain of acidic calponin (*35*).

Interestingly, as shown in Fig. 4, all three classes of calponin isoforms exhibit homology with the amino-terminal region of the Vav protooncogene product, which is a target signal transduction protein for JAK tyrosine kinases (*63*), although they do not share the src-homology region for tyrosine phosphrylation. The Vav amino-terminal sequence (residues 1 to 65) normally functions as a negative regulatory domain, targets the Vav protein to particular subcellular sites, and suppresses its transforming potential (*50-52*). The conserved amino-terminal segment of calponin contains sequences for interaction with calcium-binding proteins (*64*) and phospholipids (amino acids 85 to 100 of human neutral calponin) (*49, 65, 66*). However, it does not contain an actin-binding sequence [residues 147 to 165 (*14*)]. Since the Vav sequence also shows similarity to the amino acid sequence of SM22, a member of the muscle protein family homologous to calponin (*28, 67*), the Vav-homology sequence of calponin may be involved in as yet unidentified functional roles shared by all of the members of this protein family. We will obtain a great insight into the potential involvement of the calponin isoforms in the signal transduction mechanism with the molecular genetic approach using mutant calponins lacking functional domains and/or critical amino acids such as consensus phosphorylation sites.

Finally, this newly identified non-smooth muscle isoform of calponin may provide clues as to the mechanism underlying the diverse functions of the smooth muscle cell calponin isoform. Overexpression of human basic calponin in cultured VSMC resulted in the enhanced cell-matrix adhesion (*68*). Similarly, the constitutively expressed basic calponin isoform in transgenic arteries suppressed the injury-induced proliferation of VSMC *in vivo* (*69*). Dynamic redistribution of calponin from being cytosolic to primarily surface-cortex-associated was observed in VSMC upon stimulation with an agonist activating protein kinase C (*70*). Furthermore, basic calponin from chicken gizzard induces actin polymerization at low ionic strength and inhibits the depolymerization of actin filaments (*71*). It also induces the formation of actin filament bundles (*13*). The non-smooth muscle isoform of calponin might be involved directly or indirectly in some cellular events mediated by the basic calponin isoform. The cDNA clone reported here will provide a useful tool for future functional studies on non-smooth muscle cell isoforms of calponin to clarify the physiological role of each calponin isoform. For this purpose, it would be clearly interesting to analyze animals with targetted disruption of each of the three calponin genes.

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