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M-caveolin, a muscle-specific caveolin-related protein

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Abstract Caveolae, small invaginations of the plasma membrane, are a characteristic feature of many mammalian cells. The best-characterised caveolar protein is the integral membrane protein, VIP21-caveolin. We now describe a novel homologue of VIP21-caveolin, M-caveolin, which is expressed exclusively in muscle. M-caveolin was shown to be expressed in differentiated myotubes but not myoblasts. Epitope-tagged M-caveolin expressed in non-muscle cells was targetted to surface caveolae where it colocalized with endogenous VIP21-caveolin. M-caveolin may play a specialised role in the caveolae of muscle cells.

Key words: Caveolae; Muscle; VIP21-caveolin; M-caveolin; Membrane; C2C12

1. Introduction

Caveolae are small invaginations of the plasma membrane which are a particularly abundant and characteristic feature of endothelial cells, smooth muscle cells, adipocytes, and fibroblasts [1]. Their exact role in these different cell types is still unclear but proposed functions include signal transduction, transcytosis across endothelia, potocytosis, and endocytosis [2,3,4,5]. Caveolae were defined morphologically by early electron microscopists as 60–80 nm diameter flask-shaped invaginations of the plasma membrane [6,7]. In contrast to clathrin-coated pits they show no clear cytoplasmic coat in conventional resin-embedded specimens. However, other techniques have revealed a striated coat on the cytoplasmic surface of the caveolae [8,9]. The coat may consist at least partly of an integral membrane protein called VIP21-caveolin ([9,10] here termed V-caveolin). This protein, which was identified independently as a v-src kinase substrate [11] and as a component of the Trans Golgi Network (TGN) and TGN-derived vesicles in epithelial cells [12,13] is highly expressed in those tissues with abundant caveolae (smooth muscle, adipose tissue and lung; [14]) and appears to be a common constituent of caveolae. V-caveolin shows an unusual topology forming a hairpin loop in the membrane with both the N- and C-termini facing the cytoplasm [13].

The exact function of V-caveolin remains unclear but recent studies give some insights. V-caveolin monomers self-associate in the ER membrane shortly after synthesis to form oligomers [15]. At a later step in the biosynthetic pathway the oligomers become insoluble in non-ionic detergents. V-caveolin has also been shown to bind cholesterol with high affinity, the bound cholesterol even resisting removal by treatment with SDS [16]. These properties of V-caveolin may be important in the formation of caveolae and the maintenance of caveolar morphology and function. Cholesterol appears to be essential for caveolar function as shown by treatment of cells with cholesterol-binding agents [17] and may be recruited by V-caveolin. In addition, treatment of cells with cholesterol oxidase causes redistribution of V-caveolin from the cell surface [18]. Our recent results demonstrate a direct role for V-caveolin in caveolae formation. Expression of V-caveolin in a lymphocyte cell line which lacks caveolae (as defined morphologically and by the lack of V-caveolin, [19]) causes de novo formation of plasma membrane invaginations which are indistinguishable from caveolae [20].

Two isoforms of V-caveolin of different size and charge have been identified by Western blotting of 2D gels [12]. Rather than being different gene products the shorter form appears to be the result of translation from an inner initiation site [21]. We now describe the characterisation of a novel homologue of V-caveolin which is muscle specific. In addition database searches reveal other caveolin-related sequences. Our results suggest that a number of caveolin-related proteins have evolved to play specialised roles in different tissues.

2. Experimental

2.1. Isolation of V-caveolin homologue probe from rat

A forward primer RORfor 5′ CGCGGATCCATGTCCCTGGACTCACCCCCAGGATTT 3′ and a reverse primer RORrev 5′ CCGGAATTCTTAGCCTTCCCTTCGCAGCACCACCTT 3′ were designed based on sequence comparisons between available caveolin sequences and the 3′ UTR of the rat oxytocin receptor. These primers were used to amplify a 450 bp product, ROR1, from rat genomic DNA by PCR using the following cycling conditions, 94°C 1.0 min followed by 30 cycles of 94°C 15 s, 68°C 15 s, 72°C 15 s and 72°C 3 min. The resulting product was cloned into the *Bam*HI and *Eco*RI sites of bluescript SK II using the restriction sites introduced by the primers during the PCR reaction. The sequence of three clones, each derived from an independent PCR reaction, were determined on both strands using the T7 and T3 primers.

2.2. Northern analysis

Multiple tissue northerns (MTN) were obtained from Clonetech, Palo Alto, CA. The rat MTN was probed with the 450 bp ROR1 genomic insert that had been labeled with ^{32}P using the Prime-It labeling kit (Stratagene, La Jolla, CA) as described previously [22]. Subsequently, a mouse MTN was probed with the complete 1000 bp insert of M-caveolin using identical methods.

2.3. Isolation of mouse M-caveolin cDNA

A skeletal muscle cDNA library was constructed with the Great Lengths cDNA Synthesis Kit (Clonetech, Palo Alto, CA) using 5 μg of mouse skeletal muscle poly A+ RNA (Clonetech, Palo Alto, CA). The resulting cDNA was cloned into the *Eco*RI site of Lambda Zap II (Stratagene, La Jolla, CA) and packaged in vitro (Gigapack II Plus: Stratagene, La Jolla, CA).

The 450 bp rat genomic insert, ROR1, was labeled with the DIG random prime kit (Boehringer Mannheim, Germany) and used to probe 150,000 plaques of the mouse skeletal muscle library using standard hybridization conditions [22]. Hybridized filters were subsequently processed using the DIG system after overnight hybridization according to the manufacture's instructions (Boehringer Mannheim, Ger-

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many). The initial screen yielded three positives, B, C and E, that rescreened to homogenity. All clones were excised from Lambda Zap II into Bluescript in vivo according to the manufacturer's instructions (Stratagene, La Jolla, CA).

The complete sequences of all three clones on both strands were obtained using the T7 and T3 primers, two forward (420F TGCTACCGCCTGTTGTCT and 770F TCCTCTCAATTCCACCTC) and two reverse (310R TCCGCAATCACGTCTTCA and 570R GCGAAGAGCGGGTTGCAG) internal primers. Assembly and analysis of the M-caveolin sequences was achieved using the DNASTAR software package (DNASTAR Inc, Madison, WI). Database sequence searches were run using BLAST [23] and TBLASTN.

2.4. Differential Northern analysis

Undifferentated mouse muscle myoblast C2C12 cells were maintained at less than 50% confluence in DMEM supplemented with 10% fetal calf serum. The C2C12 cells were induced to form myotubes by growing 100% confluent cells in complete DMEM medium supplemented with 2% Horse Serum [24]. The cells were fed everyday for up to 7 days and myotube formation assessed by visual inspection. Total RNA was isolated from equivalent amounts of undifferentated and differentiated cells using the total RNA isolation kit (Stratagene, La Jolla, CA) while Poly A+ RNA was selected using the Poly(A) quik mRNA isolation kit (Stratagene, La Jolla, CA). 1 mg of mRNA from undifferentiated and differentiated cells was separated on a 1% formaldehyde agarose gel and processed as previously described [22]. The blot was subsequently probed with the complete M-caveolin cDNA.

2.5. Expression of M-caveolin in HeLa cells

M-caveolin was cloned into CB6KXHA, a derivative of the transfection vector CB6 [25], which contains the HA tag, YPYDVPDYA immediately downstream of an in-frame *Not*I site (Higley and Way, unpublished). Briefly, an in-frame *Not*I site was inserted at the C-terminus of M-caveolin by PCR using the reverse primer 5′ GGGGCGGCGGCCGCCGCCTTCCCTTCGCAGCACCAC 3′ in conjunction with the T7 vector primer. The resulting product was cloned into the *Hin*dIII–*Not*I sites of CB6KXHA using a unique *Hin*dIII site at base pair 37 in the 5′ UTR of M-caveolin and the *Not*I site engineered by PCR. The sequence of the final construct CB6M-cavHA containing M-caveolin with an in-frame HA-tag at the C-terminus was confirmed by sequencing both strands. HeLa cells were grown until approximately 50% confluent. They were incubated with 1 μg of the M-caveolin-HA construct in serum-free medium for 1 h and then 4 μl of lipofectin reagent (Boehringer-Mannheim, Germany) was added. After a further 4 h the cells were washed and further incubated in serum-containing HeLa medium. Transfection experiments were analysed by immunofluorescence between 15 and 36 h later. Cells were fixed with 3% pfa in PBS and permeabilised with 0.1% saponin. After quenching with 50 mM NH_4Cl they were incubated in 0.1% BSA, 0.5% fish skin gelatin for 30 min and then with the primary antibodies in PBS containing 0.05% saponin. V-caveolin was detected using antiserum or affinity purified antibodies against an N-terminal peptide [13] and HA-tagged M-caveolin with a mouse monoclonal to the HA epitope (12CA5, Boehringer).

For immunoelectron microscopic localisation of expressed M-caveolin, HeLa cells cultured in a 6 cm dish were transfected with HA-tagged M-caveolin as described above. After 36 h they were fixed with 3% paraformaldehyde in 100 mM phosphate buffer, pH 7.35, for 30 min at room temperature and then scraped from the culture dish and pelleted. Processing for frozen sections, sectioning, and immunolabelling were as described previously [13].

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CCTATTTAGCCGGCAGCGGCACCAGTCTGGAGACCCTAAGCTTGGTCTTTCTGCCCCCAG   60
GACTATCAACGATACCAGCCACAAGGCTCTGATCGCCTCCTGAAGGAGCCCCAGCCTCAC  120

  M  M  T  E  E  H  T  D  L  E  A  R  I  I  K  D  I  H  C  K   20
AATGATGACCGAAGAGCACACGGATCTGGAAGCTCGGATCATCAAGGACATTCACTGCAA  180
                                                  -  -
  E  I  D  L  V  N  R  D  P  K  N  I  N  E  D  I  V  K  V  D   40
GGAGATAGACTTGGTGAACCGCGACCCCAAGAACATCAATGAGGACATTGTGAAGGTAGA  240
                                                  R  -
  F  E  D  V  I  A  E  P  E  G  T  Y  S  F  D  G  V  W  K  V   60
TTTTGAAGACGTGATTGCGGAGCCCGAGGGCACCTACAGCTTCGACGGTGTATGGAAGGT  300
  -  Y  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
  S  F  T  T  F  T  V  S  K  Y  W  C  Y  R  L  L  S  T  L  L   80
GAGCTTCACCACGTTCACCGTCTCCAAGTACTGGTGCTACCGCCTGTTGTCTACACTGCT  360
  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
  G  V  P  L  A  L  L  W  G  F  L  F  A  C  I  S  F  C  H  I  100
GGGTGTTCCACTGGCCCTGCTCTGGGGATTCCTGTTCGCCTGTATCTCCTTCTGCCACAT  420
  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
  W  A  V  V  P  C  I  K  S  Y  L  I  E  I  Q  C  I  S  H  I  120
CTGGGCCGTGGTGCCCTGCATTAAGAGCTACCTGATCGAGATCCAGTGCATCAGCCACAT  480
  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
  Y  S  L  C  I  R  T  F  C  N  P  L  F  A  A  L  G  Q  V  C  140
CTACTCACTGTGTATCCGCACCTTCTGCAACCCGCTCTTCGCTGCGTTGGGCCAGGTCTG  540
  -  -  -  -  -  -  -  -  -  -  -  -
  S  N  I  K  V  V  L  R  R  E  G  *                          151
CAGCAACATTAAGGTGGTGCTGCGAAGGGAAGGCTAAAGCCAGGCTGGGCTGTGGGGAAG  600

GCTGGGCAGGGGGATCAAAGGTCAGGTGAGCATACCTGGCTCTGCTCCACATGGGCTGCT  660
GGAGAGCTCTTCCTTTTGAGGTTCCTCTCAATTCCACCTCAAGATGGGAAAACTTGGTGC  720
GGGAGGAGCCAGAAGGAAAAGACAGCCCAGTGTGGAAACACGGGCACCCCTGCTCCGCTC  780
AGCCTCCCATGAACCCCGGCGGCCCTGACTGAGCGGAGGGTTCCTTAAGAGGCAGCTAGC  840
GCAAGGCTTTGCGTTCACATGTACTGTAATAGCCAGTCTTCTAGAGGTGTGACTCGTCTC  900
CTCATGCTAGGGGGAGCAAGCAGTGACTGCCGGCATTGAGGAAGGTGCTGCAATAAAGGG  960
TTTTAACTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                     1000
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Fig. 1. Nucleotide and amino acid sequence of M-caveolin. The nucleotide sequence of mouse M-caveolin together with its deduced amino acid sequence (bold). The polyadenylation signal at the 3′ end of the DNA sequence is shown in bold. Identical amino acid residues of the rat ROR1 probe are indicated by horizontal lines and the two conserved substitutions are shown above. The sequence of M-caveolin has been deposited in GenBank, accession number U36579.

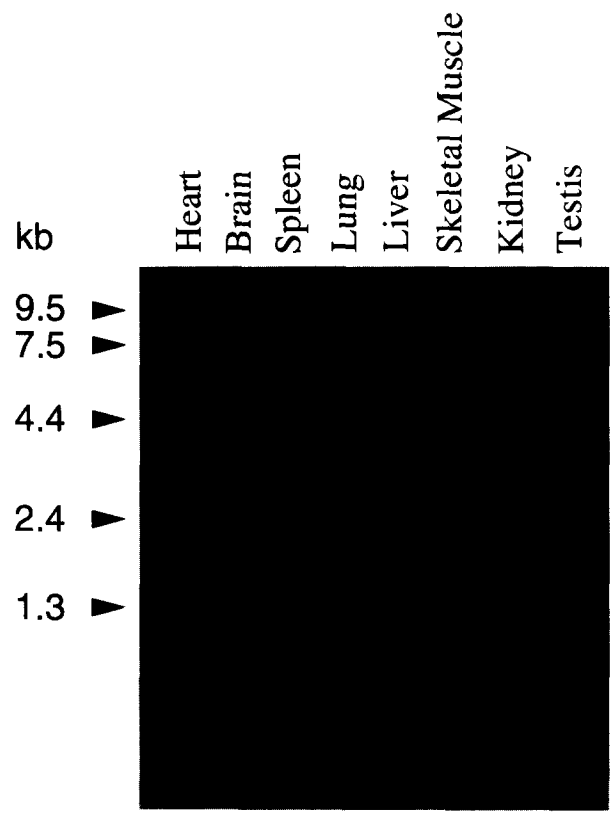

Fig. 2. Tissue-specific expression of M-caveolin. Expression of mouse M-caveolin was examined by Northern blotting of mRNA from the indicated tissues. Overnight exposure shows a single abundant transcript of approximately 1 kb in heart and skeletal muscle. A similar size but fainter band is also evident in lung.

3. Results

3.1. Isolation of a V-caveolin homologue

Database searches using the BLAST programme revealed possible homology to the V-caveolin gene in the non-coding strand of the 3′ untranslated region of the rat oxytocin receptor (ROR). Primers corresponding to the predicted 5′ and 3′ ends were used amplify a single 450 bp band by PCR from rat genomic DNA. The sequence of a single clone from three independent ROR reactions was determined. All three sequences

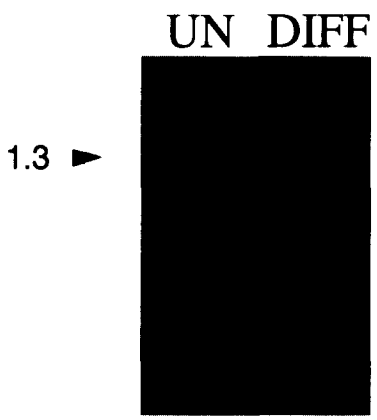

Fig. 3. M-caveolin expression is induced upon muscle differentiation. mRNA from undifferentiated (UN) and differentiated (DIFF) C2C12 cells probed with the M-caveolin cDNA reveals that the message is only expressed in myotubes. Lanes contain equal loadings.

showed the predicted similarity to V-caveolin except that the frame shifts seen in the original rat genomic DNA were absent. Northern analysis with ROR1 on a rat multiple tissue Northern detected a single band of ~1.0 kb in heart and skeletal muscle (data not shown). Subsequently, ROR1 was used to probe and isolate three independent clones from a mouse skeletal muscle cDNA library. The sequence of the three clones were found to be identical and encoded a protein of 151 amino acid residues with a predicted mass of 17.3 kDa (Fig. 1). We are confident that the position of translation is correct as multiple stops exist in the sequence immediately 5′ to the initiator Met. The deduced sequence of the mouse clone showed only two conservative changes from the sequence of the three ROR clones derived from the rat genomic DNA (Fig. 1).

3.2. V-caveolin homologue is muscle specific

We examined the expression of the caveolin-like gene by Northern blotting of multiple mouse tissues. A single band of ~1.0 kb was detected in skeletal muscle and heart (Fig. 2). A very faint band was also seen in lung. This may be derived from small amounts of smooth muscle tissue that would be expected to be present in the lung tissue. No band was detected in the other tissues tested even after longer exposure times. To further confirm the muscle specificity we probed Northern blots of undifferentated and differentiated mouse muscle myoblast C2C12 cells. Even with long exposure times a ~1.0 kb could only be detected in cells that had been differentiated into myotubes (Fig. 3). Based on its muscle-specific expression, we have termed the V-caveolin homologue M-caveolin for muscle-specific caveolin.

3.3. Sequence comparison of M- and V-caveolin

The sequence of M-caveolin is 64.2% identical to V-caveolin (Fig. 4). Like V-caveolin, M-caveolin contains a 33 amino acid hydrophobic domain, residues 74–107, that is predicted to form a hairpin loop in the membrane with both the N- and C-termini facing the cytoplasm. The conservation of the length and sequence of this domain suggests it plays an important role in caveolin targeting and/or function while the more variable C-terminus may confer muscle-specific functions. In addition, database searches with the M- and V-caveolin protein sequences identify four human expressed sequence tags, yl35d07.s1, ye79f05.r1, yd37h10.r1 and yj31h07.s1, that contain homologous but not identical sequences, suggesting the existence of a third caveolin-related protein.

3.4. Expressed M-caveolin is associated with caveolae

We examined whether M-caveolin contains targetting information for caveolar localisation by expressing an epitope-tagged M-caveolin in fibroblasts. HeLa cells were transfected with a construct containing M-caveolin with a C-terminal HA tag. The expressed protein, localized using antibodies to the HA tag, showed almost complete colocalisation with the endogenous V-caveolin, detected using antibodies to its N-terminus (Fig. 5). Cells treated identically but without addition of DNA showed no labelling with the HA antibody. We then examined the localisation of the tagged protein by immunoelectron microscopy on frozen sections. Specific labelling was found on plasma membrane-associated caveolae with no labelling of the intervening plasma membrane (Fig. 6). M-caveolin, like V-caveolin, is therefore efficiently targetted to caveolae.

4. Discussion

In the present study we have characterised a novel caveolin-related protein, M-caveolin. In contrast to V-caveolin, M-caveolin shows a very restricted distribution being expressed only in muscle. In addition, the expression of M-caveolin is extremely tightly regulated only being expressed upon differentiation of myoblasts into myotubes. Database searches with the M- and V-caveolin sequences also suggest the existence of at least one additional caveolin homologue based on four overlapping expressed sequence tags. Taken together our findings show that M- and V-caveolin are members of an emerging family of caveolin-related molecules in mammalian cells.

Comparison of the known V-caveolin sequences from different species and the sequence of M-caveolin allow us to distinguish potentially important conserved motifs from more variable regions which may have tissue-specific functions. The N-terminal cytoplasmic domain of M-caveolin is 27 amino acids shorter than that of V-caveolin. However, excluding the

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M-cav                                    MMTEEHTDLEARIIKDIHCKEIDLVNRDPKNINEDIVKVDFEDVIAEPEGTYSFDGVWKV   60
                                          *  * *         * * ***********  * * ** ************ **** **
V-cav   MSGGKYVDSEGHLYTVPIREQGNIYKPNNKAMADEVTEKQ--VY-DAHTKEIDLVNRDPKHLNDDVVKIDFEDVIAEPEGTHSFDGIWKA   87

M-cav   SFTTFTVSKYWCYRLLSTLLGVPLALLWGFLFACISFCHIWAVVPCIKSYLIEIQCISHIYSLCIRTFCNPLFAALGQVCSNIKVVLRREG  151
        ******* *** ******  * * ** **  **  ** ********** ********  **    *** *** * *   ***      *
V-cav   SFTTFTVTKYWFYRLLSTIFGIPMALIWGIYFAILSFLHIWAVVPCIKSFLIEIQCISRVYSIYVHTFCDPLFEAIGKIFSNIRISTQKEI  178
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Fig. 4. Sequence alignment of caveolin family members. Sequence alignment of M-caveolin (M-cav, top) and VIP21-caveolin (V-cav, below). Asterisks indicate identical residues. The stretch of residues shown in bold correspond to the conserved 33 amino acid stretch postulated to form the intramembrane domain. In addition, the internal initiation site (Met 33) in V-caveolin is indicated.

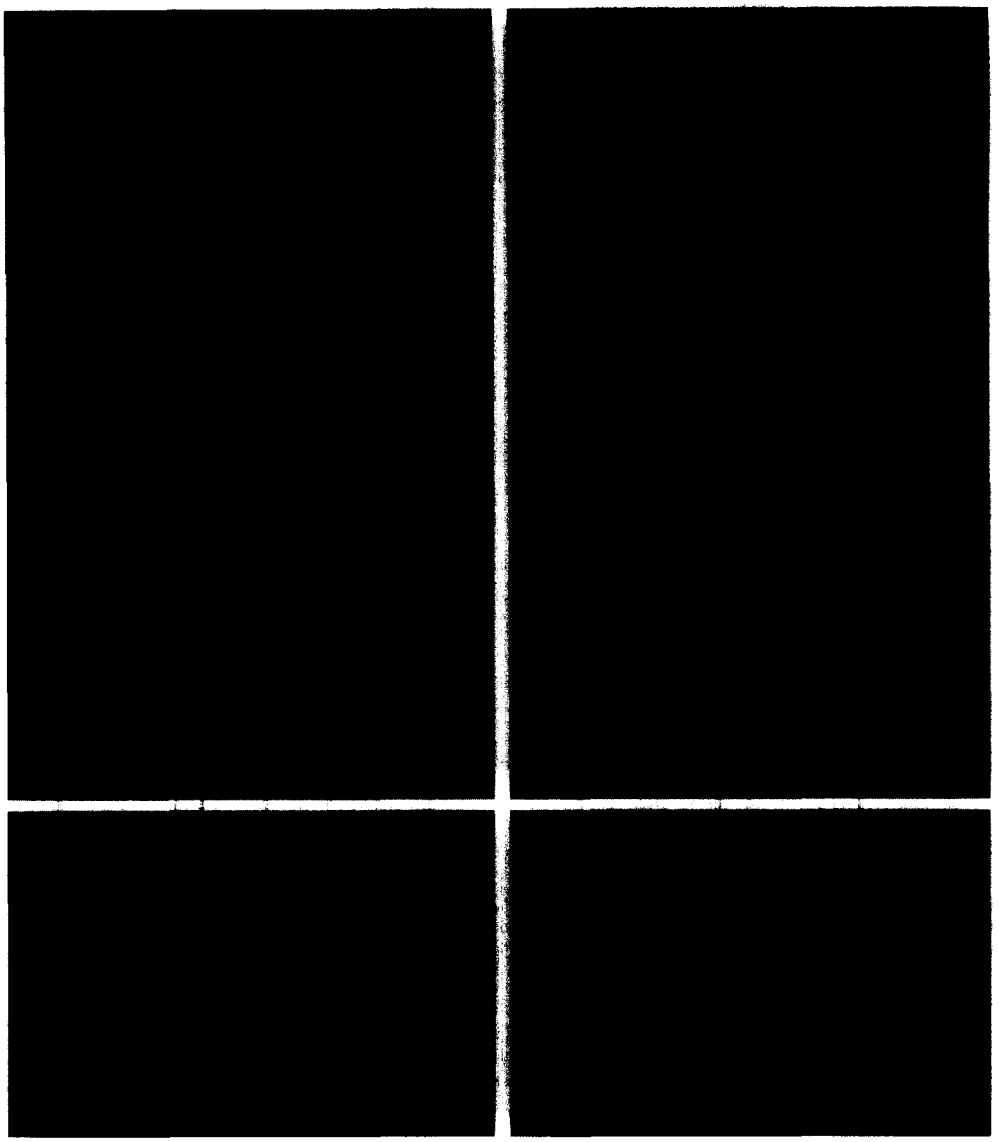

Fig. 5. Localisation of HA-tagged M-caveolin in transfected HeLa cells. HeLa cells were transfected with HA-tagged M-caveolin and then double-labelled with antibodies against the HA-tag (A and C) and VIP21-N (B and D). Note the high degree of colocalisation between the tagged M-caveolin and V-caveolin (arrows). Bar = 1 μm.

first 15 amino acid residues of M-caveolin they are 84% identical up to the putative intramembrane domain (Fig. 4). The internal initiation site for translation [21] and the conserved consensus protein kinase C (PKC) phosphorylation site of V-caveolin [26] are lacking in M-caveolin. The first region where the sequences show a high level of identity starts at the KEI (Lys-Glu-Ile) sequence (residue 20 of M-caveolin, residue 47 of V-caveolin) and extends beyond the putative intramembrane domain. Within the N-terminal cytoplasmic portion of this conserved segment lies a region of potential importance for the function of V-caveolin. Residues 61–101 have recently been shown to mediate oligomerization of V-caveolin [27] and residues 82–101 to interact specifically with trimeric G-protein subunits [28]. Part of the latter region also shares homology with a conserved motif in Rab GDI proteins [28]. It therefore seems likely that M-caveolin also interacts with trimeric G-proteins.

The putative intramembrane domains of V-caveolin and M-caveolin are also highly conserved. By analogy to V-caveolin, we presume that this domain of M-caveolin also forms a hairpin structure in the membrane with both the N- and C-termini facing the cytoplasm [10]. The high degree of homology within this domain, and the lack of similarity to intramembrane domains of other membrane proteins, suggests a crucial role in membrane integration or in interaction with specific membrane components (see [29]). These interactions may be essential for targetting to, or formation of, caveolae [20]. The C-terminal cytoplasmic domains of M-caveolin and V-caveolin are the same length and show a high degree of similarity but are only 55% identical. M-caveolin contains several cysteine residues which may be sites for palmitoylation as already shown for V-caveolin [30]. In particular Cys^{116} and Cys^{129} of M-caveolin, which correspond to Cys^{143} and Cys^{156} of V-caveolin, are in highly conserved regions.

The most striking finding of the present study is the restricted tissue distribution of M-caveolin. Northern analysis showed

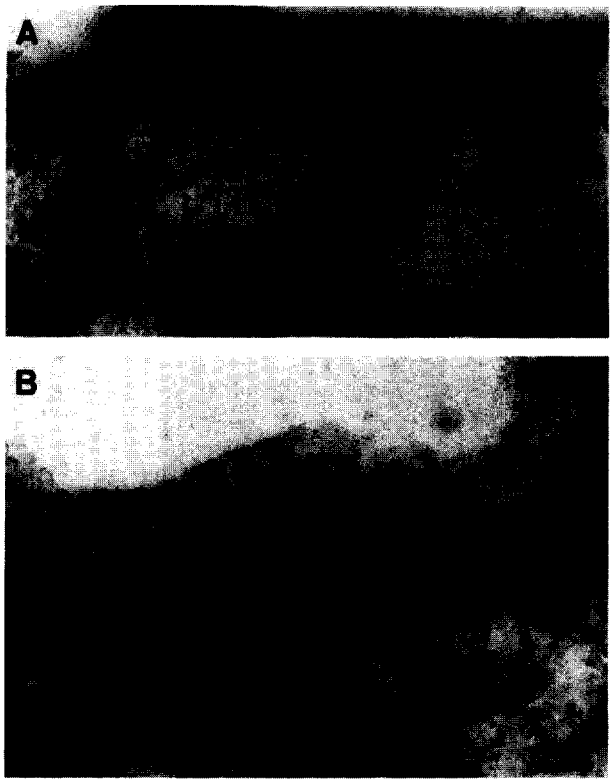

Fig. 6. Immunoelectron microscopic localisation of expressed HA-tagged M-caveolin in HeLa cells. HeLa cells were transfected with HA-tagged M-caveolin and then processed for frozen sectioning. Sections were labelled with antibodies to the HA tag followed by 10 nm protein A-gold. Specific labelling is associated with caveolae (arrowheads) but not with the intervening plasma membrane. Double arrowheads indicate a clathrin coated pit for comparison. Bars = 100 nm.

that M-caveolin is only present in muscle tissue and that it is induced upon differentiation of C2C12 muscle cells. From previous work it appears that V-caveolin is also expressed in muscle [14,31] but does not show the same restricted expression pattern as M-caveolin. The muscle-specific expression of M-caveolin provides a powerful system in which the function of this protein can be examined through knockout mice and dominant negatives or antisense in differentiating C2C12 cells. Our studies show that in fibroblasts M-caveolin and V-caveolin are targetted to the same caveolae. Thus M-caveolin has the appropriate signals for targetting to caveolae. Localisation of M-caveolin and V-caveolin at the ultrastructural level in muscle will now be required to see whether different subsets of caveolae exist in vivo. While caveolae of non-muscle cells have been implicated in diverse functions such as potocytosis [3], transcytosis [32] and signal transduction [4] their function in muscle is still unclear. In smooth muscle cells caveolae, defined morphologically and by V-caveolin labelling, are concentrated in specific areas of the sarcolemma in dystrophin-rich domains [33]. In skeletal muscle caveolae are abundant close to the T-tubules [34,35]. In view of the observed concentration of calcium-regulating molecules in caveolae of different cell types including smooth muscle [36,37] it is possible that muscle caveolae play some specific role in calcium signalling [35]. The further characterisation of M-caveolin should provide important insights into the role of caveolae in muscle cells.

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