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 targeted 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, podocytosis, 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 caveolar 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 from rat

A forward primer RORfor 5' CCAGGATCCATGTCCCTGGAC- TCACCCCCAGGATT 3' and a reverse primer RORrev 5' CCGG- AATTCTTACCCCTTCGAGCCACCCCTT 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 BamHI site of Lambda Zap II and packaged in vitro (Gigapack II Plus: 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.

2.2. 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 32P using the Prime-It labeling kit (Stratagene, La Jolla, CA) and used to probe 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 manufacturer's instructions (Boehringer Mannheim, Ger-

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The complete sequences of all three clones on both strands were obtained using the T7 and T3 primers, two forward (420F TGGCTAC- CGCGCTTGTGTCT and 770F TCTTCTCAATTCACCCCTC) and two reverse (310R TCGCGAATCAGCTTCTAC and 570R GCGAAGAGG-GGAGTTGAC) 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. Differential Northern analysis

Indifferentiated 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 every day for up to 7 days and myotube formation assessed by visual inspection. Total RNA was isolated from equivalent amounts of undifferentiated 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. 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 NotI site (Higley and Way, unpublished). Briefly, an in-frame NotI site was inserted at the C-terminal of M-caveolin by PCR using the reverse primer 5’ GGGGCGGCGGCGCGCCCTTCTCCCTGCGACGAGACACTACAGTAGC 3’ in conjunction with the T vector primer. The resulting product was cloned into the HindIII-NruI sites of CB6KXHA using a unique HindIII site at base pair 37 in the 5’ UTR of M-caveolin and the NruI site engineered by PCR. The sequence of the final construct CB6M-caveolin 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 NH4Cl 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 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).

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
expressed in myotubes. Lanes contain equal loadings. ~1.0 kb was detected in skeletal muscle and heart (Fig. 2). A very specific caveolin.

3.2. V-caveolin homologue is muscle specific
We examined the expression of the caveolin-like gene by 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 three ROR clones derived from the rat genomic DNA (Fig. 1).

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, yl3sd07.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 targeting 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 intramembrane domain. In addition, the internal initiation site (Met 33) in V-caveolin is indicated.
Fig. 5. Localization 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 colocalization 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 targeting 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 Cyssuperscript 116 and Cyssuperscript 129 of M-caveolin, which correspond to Cyssuperscript 143 and Cyssuperscript 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
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 targeted to the same caveolae. Thus M-caveolin has the appropriate signals for targeting 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.

References


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