Differential actin organization by vinculin isoforms: implications for cell type-specific microfilament anchorage

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Abstract Vinculin is found in all adherens junctions, while metavinculin, a larger splice variant, is coexpressed with vinculin only in smooth and cardiac muscle. To understand the significance of metavinculin expression, we compared ligand binding between turkey vinculin and metavinculin. Residues 1–258 were found essential for head-tail interactions in both proteins. The tail domains (VT and MVT, respectively) both bind to F-actin. However, while VT bundles F-actin, MVT generates highly viscous F-actin webs. In transfected PtK2 cells, VT causes F-actin needles or coils, while MVT-expressing cells display a diffuse F-actin distribution. Thus, the MVT-specific insert induces an F-actin supraorganization different from the VT-based form, suggesting that metavinculin has a specific role in muscle.

Key words: Vinculin; Metavinculin; Actin organization; Myofibril anchorage

1. Introduction

Vinculin plays a crucial role in linking microfilaments to the cytoplasmic face of the plasma membrane in many different cell types [1–3], and disruption of the single vinculin gene is lethal for the development of nematodes [4] as well as for vertebrates [5]. The 116-kDa protein consists of a large 90-kDa globular head domain and a rodlike 29-kDa tail domain that can be separated by endoproteinase GluC (V8) cleavage [6,7]. Vinculin is a multi-ligand protein which can interact with talin, α-actinin, paxillin, acidic phospholipids, F-actin (for review, see [8]) and with the cell contact protein VASP [9–12]. Interactions between the head and tail domains [13] can fold the molecule [14] and thus influence ligand binding [15–17] and this head-tail association is regulated by acidic phospholipids [12,18–20] and possibly phosphorylation [21].

In vertebrate muscle, metavinculin, a larger splice variant, is coexpressed with vinculin [22–27]. The difference between vinculin and metavinculin resides solely in an insert within the tail domain, which comprises 68 or 69 amino acids in porcine or chicken smooth muscle, respectively, [26,27] and 79 amino acids in *Xenopus* [28]. Except for a small amount detected in platelets [29], metavinculin has only been found in muscle tissue. Smooth muscle and cardiac muscle are especially rich in this vinculin isoform, and some smooth muscle cells, like those of porcine stomach and blood vessels, express more metavinculin than vinculin [30]. Immunofluorescence with antibodies discriminating between metavinculin and vinculin revealed that both proteins colocalize in the same adhesion site, i.e. in the membrane-apposed attachment plaques of smooth muscle cells, and in costameres and intercalated discs of cardiocytes [31]. These findings, together with the overall structural similarity between metavinculin and vinculin molecules [7,30], have stimulated speculations that these two proteins have a similar, if not identical function in linking actin filaments to the plasma membrane [30]. Consistent with this view, no ligand for the metavinculin-specific insert has been identified. On the other hand, deficiency in metavinculin is associated with a particular form of dilated cardiomyopathy in man [32], suggesting a specific role for this splice variant in heart muscle.

To characterize intramolecular flexibility and ligand binding in metavinculin, we mapped the binding sites of the vinculin and metavinculin tail within the vinculin head. Both tail structures bind to the same sequence in the vinculin head with similar affinities. In addition, we compared the F-actin binding ability of the metavinculin tail (MVT) with that of the vinculin tail (VT). Using recombinant proteins, we show that the F-actin binding domain in MVT is functional, but MVT-actin complexes display an actin filament supraorganization which differs grossly from that induced by VT, in vitro and in transiently transfected PtK2 cells.

2. Material and methods

2.1. RT-PCR and expression cloning in E. coli

Total RNA was purified from turkey gizzard [33] and used for reverse transcription and polymerase chain reaction (PCR) with the following primers (see Fig. 1): VT-SE1 (ATATA*CATATG*CTTGCTCCTCCAAAA) contains an *Nde*I-site (italics) with ATG startcodon and codes for nucleotides 2572–2586 (underlined) of the vinculin coding sequence (GenBank J04126). VT-RE1 (TGGTG*CTCGAG*CTGATACCATGGGGT) contains an *Xho*I-site (italics) and is complementary to nucleotides 3184–3198 (underlined) of the vinculin coding sequence. Reverse transcription was carried out with the RT-PCR Kit (Stratagene). PCR products were cloned in frame with a C-terminal His-tag into expression vector pET23a (Novagen) to form pET23-VT and pET23-MVT (vinculin-tail and metavinculin-tail expression plasmids). Both constructs were sequenced with T7 DNA polymerase.

2.2. Protein expression, purification and analysis

pET23-VT and pET23-MVT were transformed into *E. coli* BL21(DE3). Twelve hours after induction log phase cultures by IPTG, the cells were harvested and lysed in ice-cold lysis buffer (50

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mM Na-phosphate pH 8.0, 0.3 M NaCl, 0.5 mM PMSF, 5 mM 2-mercaptoethanol) by addition of lysozyme (1 mg/ml) and French press treatment. Cleared extracts were passed over a column of 1 ml Ni-NTA resin (Qiagen) and the soluble protein was eluted with a 40-ml linear gradient (0–0.5 M imidazole) in washing buffer (50 mM Na-phosphate, 0.3 M NaCl, 10% glycerol, 0.5 mM PMSF, 5 mM 2-mercaptoethanol, pH 6.0). Fractions containing VT or MVT, respectively, were pooled, dialyzed against buffer A (50 mM Tris-HCl, pH 7.2, 20 mM NaCl, 1 mM EGTA, 1 mM EDTA, 5 mM 2-mercaptoethanol) and further purified by FPLC (Pharmacia, Uppsala, Sweeden) on a MonoS HR 5/5 cation exchange column in buffer A, and a linear NaCl gradient (0.02–0.5 M). Electrophoretically pure fractions were pooled and either stored at 4°C or drop-frozen in liquid nitrogen and stored at −80°C. Protein concentrations were either determined according to Bradford [34] or by using quantitative amino acid analysis with pre-column derivatization as described [35]. Approximately 1.5 mg of pure protein was obtained per 2 l culture. Vinculin head fragment was obtained from purified gizzard vinculin by V8 cleavage [36]. Proteins were separated by SDS-PAGE [37]. Gels were stained with Coomassie Brilliant Blue or transferred onto Immobilon P (Millipore). Blotting and immunolabeling were performed as described [38], using a monoclonal antibody (4E7) specific for an epitope shared by both VT and MVT, or a histidine-tag antibody (Dianova). N-terminal sequencing was performed as described [36].

2.3. Probing for head-to-tail interactions

Interaction of VH with VT or MVT tail domains was tested with the yeast two hybrid system [39], using the Matchmaker Kit (Clontech Lab., Inc.), according to the manufacturers' instructions. cDNAs coding for VH (residues 1–850) and a variety of deletion fragments were cloned into the *Eco*RI/*Sal*I site of plasmid pGBT9. Analogously, cDNAs coding for VT (residues 858–1066), MVT (residues 858–1139) and a tail deletion clone (residues 993–1043) were amplified by PCR and cloned in frame with the activation domain of the transcription factor GAL4 into the *Eco*RI/*Sal*I site of pGAD424. For quantitative evaluation, β-galactosidase activity was assayed in solution with *o*-nitrophenyl-β-D-galactopyranoside as a substrate, according to the manufacturers' instruction. The signal obtained for VH-VT interaction was defined as 100%.

2.4. Actin binding assays

Skeletal muscle actin, obtained as described [40] and further purified by an additional gel filtration step, was polymerized in F-buffer (10 mM imidazole, pH 7.4, 1 mM ATP, 0.1 M KCl, 2 mM $MgCl_2$) and incubated with the recombinant proteins, also in F-buffer, at various concentrations. Complex formation was assessed by air-fuge sedimentation at $100\,000 \times g$ for 45 min as described [38]. Low shear viscometry was performed with a falling-ball viscometer as described in [41]. Bundling of TRITC-phalloidin-labeled F-actin by VT or MVT was monitored microscopically [41].

2.5. Transfection experiments

VT and MVT coding sequences were amplified from the prokaryotic expression vectors pET23-VT and pET23-MVT (see above) by PCR, using Pfu-polymerase (Stratagene) as above, and cloned into pcDNA-BiP to obtain the eukaryotic expression vectors pcVT-BiP and pcMVT-BiP. pcDNA-BiP is a modification of the pcDNA3 vector (Invitrogene) equipped C-terminally with 10 amino acids of the birch pollen profilin sequence, specifically recognized by a monoclonal antibody [42]. PtK2 epithelial cells, grown on glass cover slips in DMEM supplemented with 10% FCS, were transfected with these probes as a Ca-phosphate precipitate. Twenty-four hours later, the medium was changed and the cells were further incubated for 48 h. They were subsequently fixed in 3.7% formaldehyde, extracted in 0.2% Triton X-100 and immunolabeled with the birch profilin antibody and a TRITC-labeled second antibody (Sigma) to detect VT and MVT, respectively. Counterstaining of F-actin and microscopy were as described [42].

3. Results

3.1. Purification and characterization of recombinant VT and MVT

Turkey vinculin tail (VT, residues 858–1066) and metavinculin tail (MVT, residues 858–1135), corresponding to the VT and MVT fragments as depicted in Fig. 1A and equipped with a histidine-tag, were expressed in a soluble form. From the bacterial lysate, they were strongly enriched by affinity chromatography on Ni-NTA resin and purified to homogeneity by chromatography on MonoS (Fig. 1B). In SDS-PAGE, VT and MVT displayed an apparent molecular mass of 27 000 Da and 38 000 Da, as compared to the calculated molecular masses of 24 289 and 31 778 Da. Identity and integrity of both fragments were therefore controlled by immunoblotting with a monoclonal antibody (4E7) recognizing VT as well as MVT, by immunoblotting with an antibody against the histidine-tag and by N-terminal sequencing and quantitative amino acid

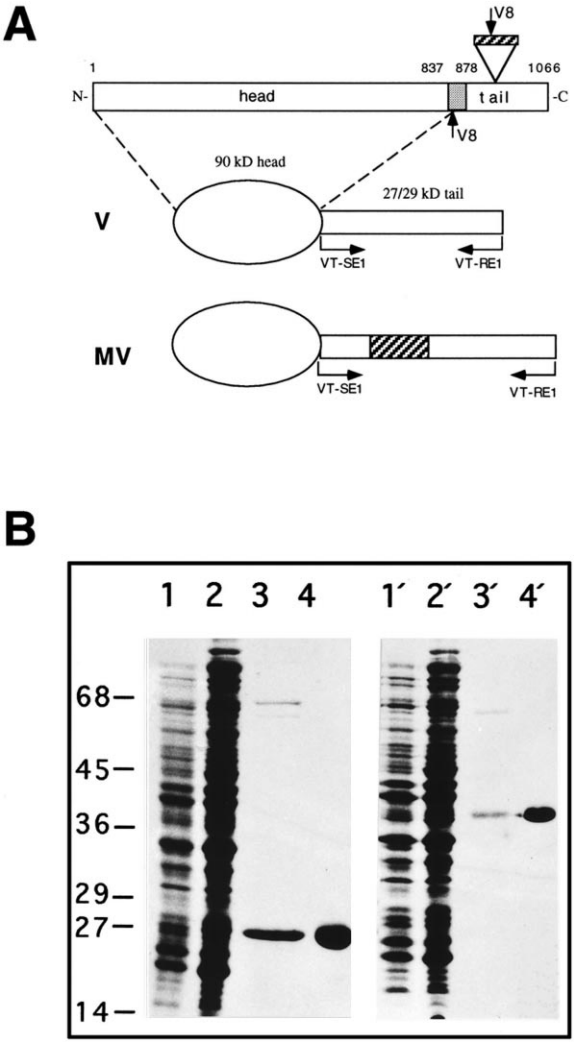

Fig. 1. A: Schematic drawing of the domain organization of vinculin (V) and metavinculin (MV). The 90-kDa N-terminal head is common to both molecules. In MV, the tail sequence contains a 69 residues long insert (hatched), C-terminal of the proline rich hinge (residues 837–878, shaded). V8 (arrows) refers to cleavage sites of the V8 protease. VT-SE1 and VT-RE1: starting points for PCR amplification of tail structures in both directions. B: Expression and purification of recombinant VT (1–4) and MVT (1′–4′). The stained SDS-PAGE profiles show bacterial lysates before (lanes 1,1′) and after (lanes 2,2′) induction, an aliquot from the pooled fractions obtained after Ni-affinity column (lanes 3,3′) and the product as purified on MonoS (lanes 4,4′). The numbers on the left refer to molecular weight markers.

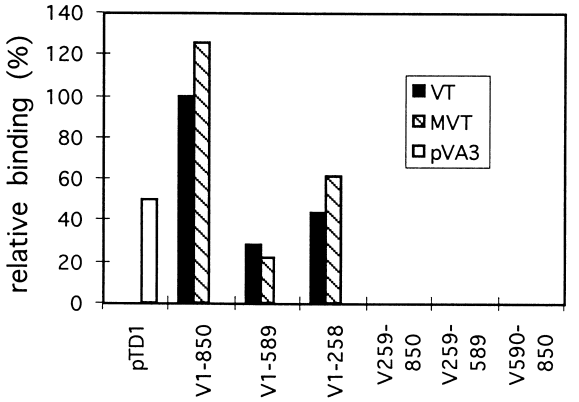

Fig. 2. Two-hybrid mapping of the VT or MVT binding site within the vinculin head. Binding of VT (black columns) and MVT (hatched columns) to various vinculin head fragments was measured in terms of β-galactosidase activity. The interaction of the full length vinculin head (V1–850) and tail (V858–1066) domains was defined as 100%. Numbers in the abscissa indicate vinculin residues in sequences expressed as fusion proteins with the GAL4-DNA-binding domain. pTD1 and pVA3: positive control sequences supplied by the manufacturer (see Section 2). Note that V1–258 is necessary and sufficient for binding.

analysis (data not shown). By these criteria, both proteins were found to be of the expected sequence and size, despite their aberrant position in SDS-PAGE. In dot overlays [20], VT and MVT interacted with VH as well as with F-actin (data not shown), demonstrating that both recombinant fragments were functional and contained intact F-actin binding sites.

3.2. Metavinculin forms intramolecular bonds like vinculin

The interaction of VT and MVT with VH was analyzed in the yeast two-hybrid system. A series of VH fragments was constructed as fusion proteins with the GAL4-DNA-binding domain in pGBT9 and tested for interaction with VT or MVT constructed as a fusion protein with the GAL4-activation domain in pGAD424. A strong interaction of both VT and MVT was observed with the vinculin head (V1–850; Fig. 2), confirming that the VH-VT interaction is of rather high affinity, with a K_d in the nanomolar range [13], and demonstrating that for MVT this is not substantially altered. By using deletion mutants, a fragment corresponding to amino acid residues 1–258 was found sufficient and necessary for the interaction of VH with VT as well as MVT.

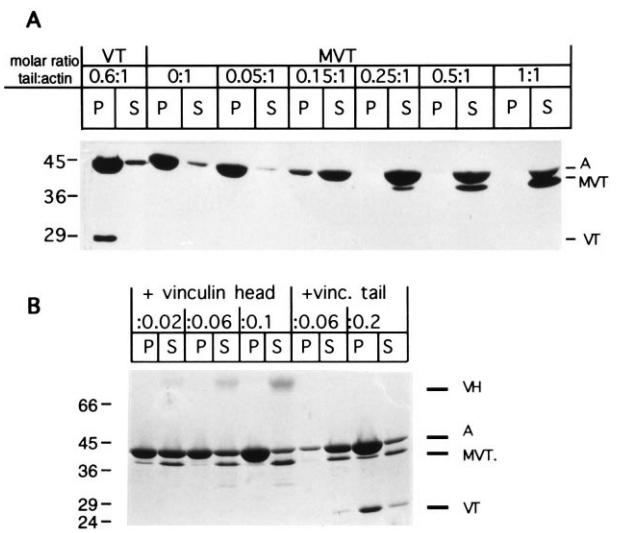

Fig. 3. SDS-PAGE profiles of pellets (P) and supernatants (S) obtained in cosedimentation assays. A: Sedimentation assays of mixtures of F-actin and MVT. Prepolymerized actin (2 μM) was mixed with VT or various amounts of MVT, in the molar ratios as indicated. VT sediments with F-actin under these conditions, while MVT interferes with actin sedimentation. B: Competitive sedimentation assay of F-actin (2 μM) with MVT (0.5 μM) and either the vinculin head (VH) or VT. The molar ratios of VH and VT to F-actin are indicated. Molecular weight markers are given on the left. The positions of vinculin head (VH), actin (A), MVT and VT are indicated on the right.

3.3. MVT-F-actin complexes do not sediment

As shown previously, genuine and recombinant VT fragments can be cosedimented with actin filaments [38,43]. Surprisingly, and despite MVT binding to F-actin had been seen in dot overlays, sedimentable MVT-F-actin complexes were not formed and F-actin did not sediment under conditions where VT-F-actin pelleted (Fig. 3). This effect could be titrated. At a molar ratio of 0.15:1 (MVT/actin), only approximately 30% of the actin was sedimentable, and above this value all actin remained in the supernatant (Fig. 3A). The influence of MVT on F-actin was studied in the presence of either vinculin head (VH) or VT (Fig. 3B). At a constant ratio of F-actin/MVT (1:0.25) increasing amounts of VH restored F-actin sedimentation, probably by binding MVT (see Fig. 2) and thus abolishing its effect on F-actin. In accordance with this, actin was found in the pellet, while MVT and VH remained in the supernatant. The addition of increasing

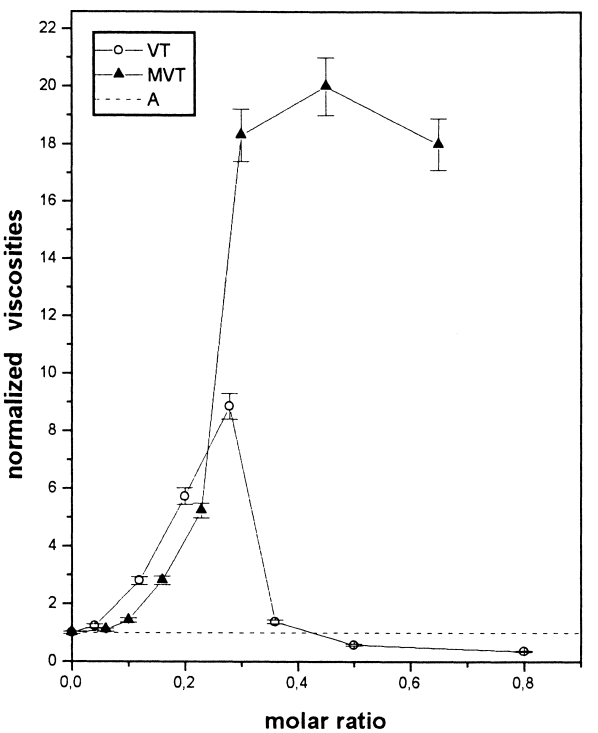

Fig. 4. Viscosity of polymerized actin (A) in the absence (dotted line) and the presence of MVT (△) or VT (○), as measured by falling ball viscometry. The viscosity of the control F-actin solution was normalized to 1, and all data were plotted in relation to this standard (normalized viscosities, ordinate). Abscissa: molar ratios of MVT and VT to 2 μM F-actin.

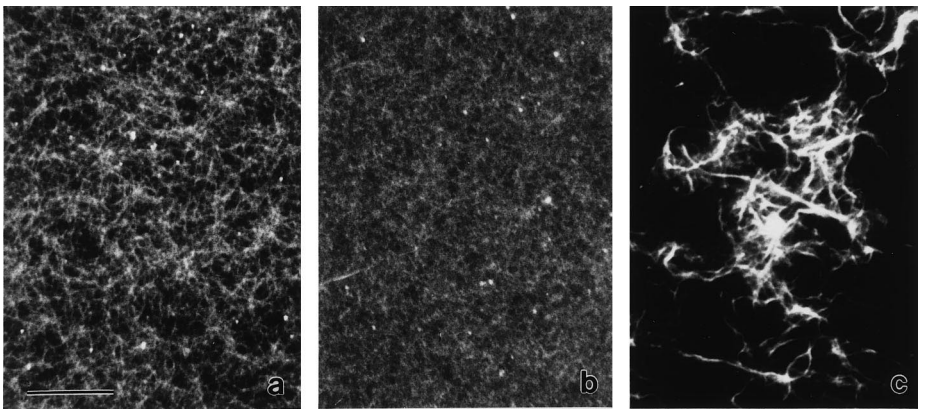

Fig. 5. Fluorescence microscopic analysis of TRITC-labeled F-actin (control), and F-actin/MVT and F-actin/VT mixtures (both at a molar MVT or VT to actin ratio of 0.6:1). While the control contains a network of rather coarse filamentous structures (a), MVT/actin mixtures reveal a much finer texture (b), and VT/actin mixtures show prominent bundles and needle-like aggregates (c). Bar = 10 μm.

amounts of VT to the same constant mixture of F-actin/MVT (1:0.25) also decreased the effect of MVT on F-actin sedimentation. In this case, VT cosedimented with F-actin, as expected, while MVT remained mostly in the supernatant, suggesting that the affinity of VT for F-actin is higher than that of MVT (Fig. 3B).

These results suggested that MVT might change the suprastructure of F-actin into an arrangement not sedimentable under the conditions chosen ($100\,000 \times g$). This hypothesis was tested by viscometry and light microscopy.

3.4. Metavinculin and vinculin tails have differential effects on F-actin viscosity

Viscometric assays were performed under low shear conditions, as described in [20,41]. At low molar ratios (up to approximately 0.3:1), VT as well as MVT strongly increased the low shear viscosity of F-actin (Fig. 4), as already reported for VT [15]. This suggests that both fragments crosslink or gelate F-actin, albeit to a different extent (Fig. 4). However, at higher molar ratios, the effects of VT and MVT on F-actin differed drastically. While MVT/actin mixtures displayed an almost constant high viscosity (Fig. 4), VT at higher concentrations caused a drastic reduction in F-actin viscosity, which fell below the level of the F-actin control (Fig. 4). The addition of VH or VT was able to reduce the MVT-induced high viscosity level to values of the control F-actin solution or even below (Table 1). Together with the sedimentation data (Fig. 3), these results indicate that MVT strongly gelates F-actin, but as MVT in solution has a higher affinity to VH and VT competes for the same binding site on F-actin, this effect can be titrated by the addition of these components. On the other hand, VT, at high concentrations, organizes F-actin into a structure of low viscosity.

Table 1
Relative viscosity of different combinations of VH, VT and MVT with actin

Component (2 μM)	Additions	Molar ratio[a]	Relative viscosity[b]
G-actin	None	–	1.0
MVT	None	–	1.1
VT	None	–	1.1
VH	None	–	1.1
F-actin	None	–	5.0
F-actin	MVT	1:0.45	23.5
F-actin	MVT+VH	1:0.45:0.2	5.5
F-actin	MVT+VT	1:0.45:0.2	7.0
F-actin	MVT+VT	1:0.45:0.9	3.0

[a]Actin/MVT/VH (or VT, respectively).
[b]The relative viscosity values were normalized to that of 2 μM G-actin = 1.0.

3.5. MVT induces actin filament webs, while VT bundles actin filaments

Using light microscopy with fluorescently labeled F-actin [41], we could visualize the different forms of actin supraorganization induced by either MVT or VT. At a molar ratio of 0.6:1 (MVT or VT/actin), the difference in viscosity between both probes was almost maximal (Fig. 4). Aliquots of these mixtures and F-actin alone were applied to a glass coverslip and examined by fluorescence microscopy (Fig. 5). While control actin filaments were rather homogeneously distributed, displaying some coarser fibers which might be parallel aggre-

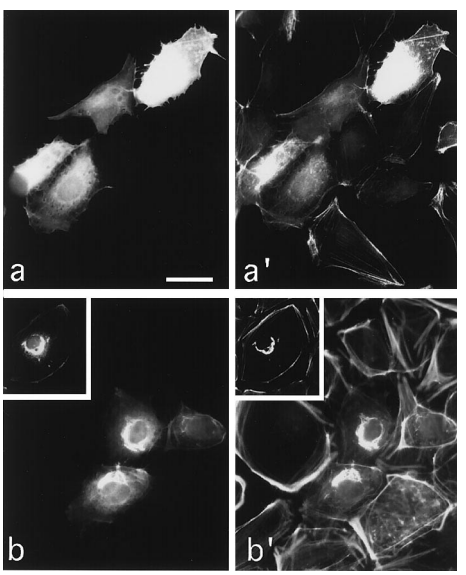

Fig. 6. Fluorescence micrographs of PtK2 cells transfected with MVT (a,a′) or VT (b,b′) constructs. The cells were stained with an antibody against the BiP-tag (a,b; see text) and counterstained for F-actin with FITC-phalloidin (a′,b′). While MVT caused F-actin to arrange in a dense web (a,a′), VT collected F-actin into large bundles or coils many of which were deposited in the perinuclear area (insets, b,b′). Bar = 20 μm.

gates (Fig. 5a), samples containing MVT revealed a much finer network or web (Fig. 5b). On the other hand, samples containing VT showed actin filaments collected into prominent bundles or needles, interspersed with areas almost devoid of filaments (Fig. 5c), as already described [41]. These results support the interpretation of the results shown in Figs. 3 and 4: high amounts of MVT cause the rearrangement of actin filaments into viscous webs which are difficult to sediment, while VT can recruit actin filaments into large needle-like structures which sediment easily but show a low viscosity, in analogy to the effect expected for a phase separation [41].

3.6. In transfected cells, MVT and VT show differential effects on actin organization

PtK2 cells were transfected with MVT or VT containing constructs, and the expressed MVT and VT proteins were monitored in total protein extracts in immunoblots (data not shown) and by immunofluorescence (Fig. 6). When the transfected cells were examined more closely, a drastic difference in the arrangement of F-actin between MVT- and VT-expressing cells became evident: in the MVT-expressing cells, actin filaments were arranged in a dense web, which also contained MVT and filled a large part of the cytoplasm (Fig. 6a,a′). In contrast, VT-expressing cells displayed densely packed aggregates of F-actin costaining for VT (Fig. 6b,b′). In many cases, these aggregates were deposited as tightly coiled bundles in the perinuclear area (Fig. 6b,b′, insets). Hence, in transiently transfected cells, the expressed MVT and VT fragments interact with filamentous actin, exerting the same pronounced, but distinct effects on actin organization as in vitro.

4. Discussion

In this study, we show that metavinculin, a splice variant of vinculin, binds to F-actin via an actin binding domain localized in the C-terminal tail, analogous to vinculin. Since the actin binding motif in avian vinculin has been assigned to sequences C-terminal of residue 893 [20,38], while the 69 residue long metavinculin-specific insert is located between residues 915 and 916, a disruption of the actin-binding site by the insertion of this rather acidic sequence stretch (p*I* 3.65, as calculated by the program Isoelectric from the GCG package) and failure of actin binding might have been expected. However, cosedimentation studies and viscosity assays clearly demonstrate a direct interaction between MVT and F-actin. Thus, the metavinculin insert does not physically interrupt the actin-binding region.

In analogy to vinculin, we found that while the isolated metavinculin tail (MVT) binds well to F-actin, the intact metavinculin, as isolated from gizzard, binds only weakly or not at all (data not shown). These data suggested that the biological activity of metavinculin is regulated by an intramolecular interaction between the head and tail domain, as had been described earlier for vinculin [13,17,19,36]. Using the yeast two hybrid system, we now mapped the vinculin and metavinculin tail binding site to the N-terminal third (residues 1–258) of the head domain, confirming earlier results obtained with GST-fusion proteins for vinculin [44]. No significant differences in the affinity of VT or MVT for VH were detected. Thus, the head might cover the F-actin binding site within the tail domains of both isoforms, and the tail domains might interfere with the binding sites of α-actinin and talin, which have been mapped to the same region of the head domain [36,45], and even with the binding of VASP to the proline-rich hinge region [9,10]. As the head-tail interactions in both cases are of high affinity, for myofibrillar anchoring, metavinculin in muscle would have to be opened and activated, possibly by oligomerization and phospholipid binding, as recently postulated for vinculin [12,18,20].

Sedimentation and viscosity assays demonstrated that MVT has a strong gelating effect on actin filaments, and these data were corroborated by direct microscopic inspection of MVT/F-actin mixtures, which displayed a fine texture of interwoven filaments, distinct from the image obtained for the F-actin control, and strikingly different from the F-actin bundles induced by VT. The data obtained with transiently transfected PtK2 cells are consistent with the in vitro effects. At present, we can only speculate why the insertion of the MVT-specific sequence into the tail counteracts this bundling activity and causes the formation of F-actin webs instead. It is conceivable that the addition of negative charges plus an increase in tail length yields a structure which has an overall lower affinity to F-actin, but is more flexible. Such a molecule might then be capable to bind at crossover points of actin filaments, preventing parallel arrangements but stabilizing a network instead.

Our results suggest that the ratio between vinculin and metavinculin at the cytoplasmic face of attachment plaques might influence the actin organization in this area. In the cell-matrix junctions of non-muscle cells, the terminal portions of actin filaments, confronted with high local concentrations of activated vinculin, would be bundled into the arrowhead-shaped focal adhesions. In the intercalated discs and attachment plaques of heart and smooth muscle, respectively, which both contain vinculin and metavinculin [31], the thin filaments do not converge but are anchored to the plasma membrane either in parallel or obliquely in a criss-cross pattern [46]. Based on the data presented here, it is conceivable that the ratio between both isoforms might determine the microfilament suprastructure. Alternatively, the muscle-specific expression of metavinculin might be correlated with the fact that attachment plaques and intercalated discs, once assembled, are permanent structures, in contrast to the highly dynamic focal contacts of non-muscle cells. Another possibility has recently been proposed by Ehler et al. [47]. These authors report on several smooth muscle-specific features in a myofibroblastic cell line (IMR-90), including the expression of metavinculin. They suggest that metavinculin might mediate the regular spacing and longitudinal arrangement of actin attachment sites seen in these cells. Further investigations are needed to discriminate between these different possibilites.

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