Metavinculin Mutations Alter Actin Interaction in Dilated Cardiomyopathy

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Background—Vinculin and its isoform metavinculin are protein components of intercalated discs, structures that anchor thin filaments and transmit contractile force between cardiac myocytes. We tested the hypothesis that heritable dysfunction of metavinculin may contribute to the pathogenesis of dilated cardiomyopathy (DCM).

Methods and Results—We performed mutational analyses of the metavinculin-specific exon of vinculin in 350 unrelated patients with DCM. One missense mutation (Arg975Trp) and one 3-bp deletion (Leu954del) were identified. These mutations involved conserved amino acids, were absent in 500 control individuals, and significantly altered metavinculin-mediated cross-linking of actin filaments in an in vitro assay. Ultrastructural examination was performed in one patient (Arg975Trp), revealing grossly abnormal intercalated discs. A potential risk-conferring polymorphism (Ala934Val), identified in one DCM patient and one control individual, had a less pronounced effect on actin filament cross-linking.

Conclusions—These data provide genetic and functional evidence for vinculin as a DCM gene and suggest that metavinculin plays a critical role in cardiac structure and function. Disruption of force transmission at the thin filament-intercalated disc interface is the likely mechanism by which mutations in metavinculin may lead to DCM.

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Key Words: cardiomyopathy • genetics • molecular biology

I diopathic dilated cardiomyopathy (DCM), characterized by dilation and impaired contraction of the heart in the absence of coronary artery disease, is a major medical problem leading to congestive heart failure, arrhythmias, stroke, and early death. Despite improved medical therapy, DCM continues to have high morbidity and mortality, and cardiac transplantation is the only definitive treatment for end-stage myocardial disease. Effective treatment and prevention is hampered by limited understanding of the pathogenetic mechanisms that cause or promote heart failure.

Recognition of DCM as a heritable disorder has stimulated efforts to discover molecular genetic defects that cause DCM. Since 1993, mutations in genes encoding cytoskeletal contractile, nuclear membrane, and other proteins have been identified in patients with familial and sporadic DCM. Although these reports clearly establish DCM as a genetically heterogeneous disorder, the molecular and cellular mechanisms by which specific mutations lead to DCM remain poorly understood.

Coordinated, synchronous contraction of the heart is facilitated by mechanical coupling between sarcomeres at Z bands, adjacent myocytes at intercalated discs, and myocytes and the extracellular matrix at costameres and dystrophin-glycoprotein complexes. We postulated that defects in proteins that mediate contractile force transmission at these sites might lead to nonuniform contraction, increased vulnerability to mechanical cellular injury and death, and progressive heart failure. Consistent with this hypothesis, mutations in genes encoding dystrophin and delta-sarcoglycan, proteins that couple contractile proteins to the extracellular matrix, and actin and tropomyosin, thin filament proteins anchored to Z bands and intercalated discs, cause DCM. Here, we further test our force transmission hypothesis by investigating vinculin as a candidate gene for DCM and functionally characterizing the molecular and cellular effects of mutations in this gene.

Vinculin is located on chromosome 10q22.1-q23 and comprises 22 exons. Exon 19 is alternately spliced with tissue-specific expression. The smaller isoform, vinculin, is ubiquitously expressed. Metavinculin, containing an additional 68 amino acids, is expressed exclusively in cardiac and smooth...
muscle. In cardiac myocytes, vinculin and metavinculin colocalize to intercalated discs and costameres. Thus, vinculin and metavinculin are located at principle sites of contractile force transmission. The identical head portions of each isoform interact with membrane-associated ligand proteins, while their unique tail segments harbor actin filament binding sites. Previous in vitro studies demonstrated differential geometric organization of actin filaments by the tail domain of each isoform, suggesting metavinculin may have a unique role in anchoring actin filaments to intercalated discs in the heart.

Methods

Study Patients

Blood samples for genomic DNA extraction and analyses were obtained from patients with idiopathic DCM after informed written consent, under a protocol approved by the Institutional Review Boards of the Mayo Clinic and University of Utah. Dilated cardiomyopathy was defined as left ventricular dimensions >95th percentile for body surface area and age, and left ventricular shortening fraction <28% and/or ejection fraction <50% determined by echocardiography. Unrelated spouses in families with autosomal dominant cardiovascular disorders served as controls.

Mutational Analyses

Oligonucleotide primers (MVCLF, 5′-CTCAGCTTTCGCCG- CATC-3′; MVCLR, 5′-CAACTGGGGGTGCTTATC-3′) were designed to amplify the coding and splice junction regions of the metavinculin-specific insert of vinculin. Sequence variants were identified by single-strand conformation polymorphism (SSCP) analyses and cycle sequencing as previously described. Nomenclature for mutations was based on metavinculin mRNA (Genbank accession No. NM_014000), with the first nucleotide of the start codon denoted as +1.

Expression Constructs

Total RNA was isolated from human heart tissue and reverse transcriptase–polymerase chain reaction (RT-PCR) was used to synthesize cDNA corresponding to the tail domains of vinculin (VT) and metavinculin (MVT), encoding amino acids 858 to 1066 and 858 to 1134, respectively. PCR primers were designed with restriction sites for further cloning into prokaryotic (pQE30, Qiagen, Hilden, Germany) expression vectors. Mutant MVT constructs, harboring each of the 3 mutations identified in DCM patients, were generated by PCR mutagenesis as described. The correct sequence of each clone was confirmed by the dideoxy chain termination technique, using the T7 sequencing kit (Pharmacia Biosystems).

Protein Isolation and Purification

All recombinant proteins bearing an N-terminal His-tag (pQE30; Qiagen, Hilden, Germany) expression vectors. Mutant MVT constructs, harboring each of the 3 mutations identified in DCM patients, were generated by PCR mutagenesis as described. The correct sequence of each clone was confirmed by the dideoxy chain termination technique, using the T7 sequencing kit (Pharmacia Biosystems).

Sedimentation Assays

The interaction of recombinant proteins with actin filaments was analyzed in a sedimentation assay. All proteins used were centrifuged (100 000×g, 3 minutes) prior to the sedimentation assay. Prepolymerized actin (3 μm/L final concentration) was incubated in the absence or presence of recombinant VT and MVT proteins at various molar ratios at 37°C for 2 hours. Samples were subjected to centrifugation at high (100 000×g, 1 hour) or low (12 000×g, 15 minutes) speed. Pellets and supernatants were analyzed by SDS-PAGE and subsequent densitometric analysis of the coomassie-stained gels.

Low Shear Viscometry and Analysis of Actin Filament Organization

Low shear viscometry was essentially performed as described. Recombinant MVT proteins were added to prepolymerized actin (3 μm/L final concentration) at various molar ratios. For in vitro analyses, recombinant MVT proteins were added to prepolymerized actin (3 μm/L) at a molar ratio of 0.6 MVT:actin. TRIC-phallolidin labeling and fluorescence analysis were as described.

Electron Microscopy

Left ventricular myocardium from the explanted heart of individual III.2 in K-4252 was fixed in formalin and embedded in paraffin at the time of cardiac transplantation. Similarly, cardiac tissue was obtained at autopsy from a 42-year-old man without cardiac pathology, 4 hours after death from a motor vehicle accident. Sections of the block with longitudinal orientation of myofibrils were selected and deparaffinized in warm xylene. Tissue was fixed in Trump’s fixative, stained with 2% uranyl, and embedded in Spurr’s resin. Thin (90-nm) sections were cut and stained with lead citrate. Multiple sections were viewed and 25 consecutive intercalated discs in each myocardial sample were qualitatively scored as “normal” or “disturbed.” Photomicrographs of representative intercalated discs were obtained on a JEOL 1200 EXII operating at 60 kV and 15 000× magnification.

Results

Vinculin Mutations Are Identified in Patients With DCM

We screened for DNA sequence variation in exon 19 of vinculin in 350 unrelated patients with sporadic and familial DCM by SSCP analysis. Mutations in cardiac actin and α-tropomyosin were previously excluded in this cohort. In addition to a common, previously reported 2814C>G neutral polymorphism (Gly938Gly), 3 unique mutations in vinculin were identified in 3 individuals. Additional mutations in the other 21 exons of vinculin were excluded by SSCP analyses. Pedigrees, results of phenotypic evaluation, and results of mutational analyses for these cases are shown in Table 1 and Figure 1. There were no associated defects of cardiac rhythm and conduction or clinically apparent noncardiac phenotypic abnormalities.

The proband in K-1702 (II.1, Figure 1a), aged 39 years, presented with shortness of breath and heart palpitations and has survived 9 years on medical therapy. By history, his father died of heart failure at the age of 59 years and a 70-year-old paternal uncle has heart failure. The proband’s relatives declined clinical and genetic evaluation. DNA sequencing of an anomalous SSCP conformer revealed a heterozygous 3-bp deletion, 2862 to 2864delGTT (data not available).
shown). This mutation creates a truncated protein by deletion of a leucine residue (Leu954del) but leaves the remaining reading frame in phase.

The proband in K-4252 (III.2, Figure 1b), aged 52 years, presented with symptomatic DCM. Although living relatives were asymptomatic, screening echocardiograms revealed DCM in a 70-year-old maternal aunt and mild left ventricular dilation in a 38-year-old daughter. Progression of heart failure in the proband led to cardiac transplantation 6 years after diagnosis. Sequencing of an anomalous SSCP conformer demonstrated a heterozygous point mutation, 2923C>T, resulting in an Arg975Trp substitution. The mutation was confirmed by testing for loss of a SfaNI restriction site, resulting in uncut 170-bp DNA fragments inherited by 2 individuals with DCM, 1 with isolated left ventricular dilation, and 1 without DCM (data not shown).

The proband in K-785 (II.1, Figure 1c), aged 30 years, was diagnosed with DCM and died of progressive heart failure 2 years later. His social history was notable for alcohol abuse. The family history was negative for DCM but relatives were unavailable for clinical and genetic testing. Sequencing of an anomalous conformer revealed a heterozygous point mutation, 2801C>T, resulting in an Ala934Val substitution (data not shown).

In addition to the other 347 unrelated DCM patients (694 chromosomes), we tested 500 unrelated control individuals (1000 chromosomes) for the 3 sequence variants identified in patients with DCM. No anomalous SSCP conformers corresponding to the Leu954del and Arg975Trp mutations were identified. However, the Ala934Val variant was found in 1 normal control, a 67-year-old woman. She had abnormal T waves on an ECG but her echocardiogram was not diagnostic for DCM. Sequence comparisons revealed that Arg975 is conserved in mouse, pig, chicken, and frog metavinculin, and Ala934 is conserved in mouse and pig metavinculin. Based on an estimated frequency of 20% for familial DCM in our cohort, metavinculin mutations occurred in 2/70 or 3% of familial cases.1

Wild-Type and Mutant Metavinculin Bind F-Actin
To investigate the effect of these mutations on metavinculin-actin interactions, constructs of the metavinculin tail (MVT) comprising the actin-binding domain were generated. Binding of recombinant wild-type human metavinculin tail (MVT wt) and the MVT mutants to filamentous actin was tested in cosedimentation assays (Figure 2a). Increasing concentra-
tions of each MVT construct were added to prepolymerized actin and actin filaments were sedimented by high-speed centrifugation. As seen by SDS-PAGE, all MVT constructs cosedimented almost quantitatively with F-actin. Densitometric analysis of Coomassie Blue–stained gels revealed that MVT binding to actin filaments was unaffected by any mutation (data not shown).

Wild-Type and Mutant Metavinculin Crosslink F-Actin

The potential influence of the 3 mutations on actin filament organization was analyzed in low-speed cosedimentation assays (Figure 2b). Whereas centrifugation at high speed (100,000g) pellets all filamentous actin and associated proteins, centrifugation at 12,000g only sediments actin filaments that are organized into F-actin suprastructures such as filamentous networks or bundles comprising cross-linked filaments. Increasing the molar ratio of any MVT construct with respect to actin caused an increase in the amount of actin that was recovered in the pellet after low-speed centrifugation, indicating that all MVT proteins were able to crosslink actin filaments. Again, no significant differences among the 4 proteins were observed in densitometric analysis of Coomassie Blue–stained gels (data not shown).

Mutations in Metavinculin Significantly Alter Actin Filament Organization

Low shear viscometry experiments allow a qualitative distinction between different types of actin-crosslinking activity. Addition of ligands that display crosslinking activity to a solution containing actin filaments alters the viscosity of the solution. The formation of filament networks increases the viscosity, whereas strong bundling causes a decrease in viscosity.

Actin filaments were polymerized in the presence of increasing amounts of MVT wt, each of the 3 MVT mutants, and vinculin tail (VT) and analyzed in a falling ball viscometer (Figure 3). Addition of MVT wt resulted in an increase in viscosity up to a molar ratio of 0.6 MVT:actin, indicating actin filament network formation. At higher molar ratios (0.8 and 1.0), the viscosity decreased again, suggesting actin bundle formation. A similar curve, albeit with lower overall changes in viscosity, was obtained for the mutant Ala934Val. In contrast, the Leu954del and Arg975Trp mutants caused a marked reduction in viscosity, reminiscent of the viscosity changes observed in the presence of VT. These data indicate that whereas MVT wt and Ala934Val organize actin filaments into networks, Leu954del and Arg975Trp induce tight actin bundles.

These observations were further supported by fluorescence microscopy. Actin filament organization by VT, MVT wt, and the 3 mutants, respectively, was directly visualized by

Figure 2. Mutations in the metavinculin insert do not affect actin binding or actin filament crosslinking. a, SDS/PAGE of pellet and supernatant fractions after high-speed centrifugation of wild-type and mutant MVT. The molar ratios of MVT construct/actin (0.1 to 0.8) are indicated; c1 represents MVT control in the absence of actin; c2, actin control. b, SDS/PAGE of actin pellets and supernatants after centrifugation at 12,000g in the presence of increasing amounts of wild-type MVT and the 3 MVT mutants, respectively. The actin control is shown in lanes “c” and molar ratios (MVT/actin) are given above the corresponding lanes.

Figure 3. MVT mutations significantly alter actin filament organization. Low shear viscometry analysis of actin polymerized in the presence of VT and MVT constructs was measured. An increase in viscosity above control levels indicates actin filament network formation. Values below the actin control value indicate strong bundling activity. Note that the differences are greatest at a molar ratio of 0.6 MVT:actin. Whereas wild-type MVT and mutant MVT Ala934Val induce network formation before bundling prevails at molar ratios above 1, mutant MVT constructs Leu954del and Arg975Trp induce only bundle formation and are more similar to the wild-type VT plot.
TRITC-labeled phalloidin staining (Figure 4). In contrast to the actin control, VT induced thick, fairly straight bundles whereas MVT wt organized F-actin into a fine filamentous network. These observations are concordant with previous data reporting differential actin filament organization by chicken VT and MVT.\textsuperscript{15} Consistent with the viscometry data, actin organization by the mutant Ala934Val was similar to that observed for MVT wt, although the network induced by Ala934Val appeared coarser. More prominent bundles were observed for the deletion mutant Leu954del. However, in comparison with VT-induced structures, the filaments tended to be longer and more flexible. Arg975Trp showed the highest bundling activity, reflected by the induction of large aggregates of actin filaments. In summary, F-actin binding is unaffected by all 3 mutations but each affects actin filament organization.

**Intercalated Discs Are Disrupted by a Metavinculin Mutation**

To identify the in situ correlates of DCM-associated mutations in metavinculin, the ultrastructure of protein complexes within cardiac myocytes was investigated by electron microscopy (Figure 5). In the control tissue, 25 of 25 intercalated discs appeared structurally normal with sharply demarcated, parallel alignment of plasma membranes of adjoining myocytes. By contrast, 17 of 25 consecutive intercalated discs in explanted heart tissue from individual III.2, K-4252 appeared irregular and fragmented. The architecture of sarcomeric thin and thick filaments appeared intact, in contrast to abnormal sarcomere structure in a DCM patient with a mutation in \( \alpha \)-tropomyosin.\textsuperscript{7}

**Discussion**

We investigated vinculin as a candidate gene for DCM, hypothesizing that it plays a central role in cardiac structure and contractile force dynamics. Vinculin and metavinculin are localized to intercalated discs,\textsuperscript{14} structures that anchor thin filaments and transmit contractile force in the heart. Vinculin expression is upregulated in response to mechanical loading,\textsuperscript{21} and targeted disruption of vinculin in mice causes loss of cardiac contractility in embryonic development.\textsuperscript{22} Moreover, human studies have suggested a potential relationship between metavinculin and vinculin expression, intercalated disc abnormalities, and DCM.\textsuperscript{23,24} Our objective in this study was to establish metavinculin dysfunction as a heritable mechanism for DCM.

The structure of the vinculin tail domain comprises a bundle of 5 \( \alpha \)-helices arranged in antiparallel orientation (Figure 6). The 3 mutations in the metavinculin-specific insert reported here are each located in a different helix and are predicted to have different effects on helical organization, consistent with the observed effects on actin organization in vitro. The conservative Ala934Val substitution had a mild effect on actin crosslinking. Deletion of Leu954 may shorten comparison with VT-induced structures, the filaments tended to be longer and more flexible. Arg975Trp showed the highest bundling activity, reflected by the induction of large aggregates of actin filaments. In summary, F-actin binding is unaffected by all 3 mutations but each affects actin filament organization.

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![Figure 4](image1.png)

Figure 4. In vitro analysis of actin filament organization by VT and MVT constructs reveals higher bundling activity of MVT mutants. Recombinant proteins were added to prepolymerized actin filaments (3 \( \mu \)mol/L) at a molar ratio of 0.6 construct/actin. a, Actin filaments alone. b, Wild-type VT organized actin filaments into short straight bundles; c, Wild-type MVT created filamentous networks. d, A coarser network is seen for MVT mutant Ala934Val. Strong bundling activity occurred with MVT mutants Leu954del (e) and Arg975Trp (f). Scale bar=10 \( \mu \)m.

![Figure 5](image2.png)

Figure 5. Intercalated discs are disrupted by the Arg975Trp substitution in metavinculin. Representative transmission electron micrographs from (a) normal heart tissue and (b) the explanted heart of individual III.2 in K-4252 (15 000× magnification) are shown. Arrows indicate intercalated discs; asterisks, Z bands.
the second metavinculin helix and thus affect the overall conformation of the insert. The Arg975Trp substitution leads to a drop in pI over almost 2 pH units, and may thus severely disturb the helix. The latter 2 mutations were shown to have a more drastic effect on metavinculin-based actin organization in vitro.

The in vitro data on actin binding are consistent with the cardiac phenotypes of patients harboring mutations in the metavinculin-specific insert. The Ala934Val substitution, showing the least pronounced alteration in actin cross-linking, was identified in a patient with DCM and a normal control individual. The effect of this rare polymorphism on metavinculin function may be insufficient to cause DCM, but together with excessive alcohol consumption, may confer additive risk for heart failure at a relatively young age. By contrast, the Leu954del and Arg975Trp substitutions have a more dramatic effect on actin organization and may also alter binding of metavinculin to phospholipids. These effects may be responsible for disruption of intercalated discs, seen in the pathological specimen from the proband in K-4252. Whether or not alteration of intercalated discs is a specific feature of mutations in vinculin will require further genetic and ultrastructural studies in patients with DCM. Whether or not disruption of intercalated discs is a ubiquitous feature of DCM? In K-4252, additional genetic and/or environmental influences clearly determine penetrance of the Arg975Trp substitution and progression of cardiac dilation to symptomatic DCM. Similar age-dependent penetrance and variable expression of DCM occurs in families with mutations in cardiac actin, desmin, and lamin A/C. Other inherited and/or acquired factors may also contribute to the pathogenesis of DCM in the patient with deletion in vinculin.

This study suggests that metavinculin plays an important role in the structural integrity and function of the heart and demonstrates that inherited dysfunction of this protein is associated with altered actin filament organization in vitro, disrupted intercalated disc structure in situ, and DCM. These findings are consistent with the hypothesis that defective contractile force transmission leads to DCM and will facilitate development of experimental models to further investigate biological mechanisms for heart failure.

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