**Chicken Vinculin and meta-Vinculin Are Derived from a Single Gene by Alternative Splicing of a 207-Base Pair Exon Unique to meta-Vinculin**

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\[meta\text{-}Vinculin\] and vinculins are closely related proteins that are cytoplasmic components of microfilament-associated cell junctions. This report describes the structural relationship between these two proteins and the genetic basis for tissue-specific expression of \(meta\text{-}Vinculin\). Analysis of genomic DNA coding for amino acids 676–1066 of vinculin revealed 9 exons spanning an 11.7-kilobase pair region of the genome. In the 4 kilobase pairs of intervening sequence that separates vinculin exons E896–E915 and E916–E984, there is an open reading frame that predicts a sequence homologous to the 68-amino acid peptide specific to porcine \(meta\text{-}Vinculin\) (Gimona, M., Small, J. V., Moeremans, M., Van Damme, J., Puype, M., and Vandekerckhove, J. (1988) EMBO J. 7, 2329–2334). Analysis of the corresponding cDNA established that chicken \(meta\text{-}Vinculin\) contains a 69-amino acid insertion between residues 915 and 916 of vinculin and that there are no other amino acid sequence differences between chicken vinculin and \(meta\text{-}Vinculin\). Muscle-specific expression of \(meta\text{-}Vinculin\) occurs by alternative splicing of a transcript produced from a single gene because: all 20 genomic isolates that contain the 3′ vinculin exons, also contain the \(meta\text{-}Vinculin\)-specific exon; Southern blots performed at high stringency with exon-specific probes indicate the presence of a single gene; and the 3′-untranslated sequences of vinculin and \(meta\text{-}Vinculin\) cDNAs are identical.

Vinculin (1) and \(meta\text{-}Vinculin\) (2, 3) are proteins that localize in the cytoplasmic plaque of microfilament-associated cell:cell and cell:substrate junctions (4, 5). In non-muscle cells, these load-bearing structures include cell-to-extracellular matrix junctions, such as focal adhesions, and cell-to-cell junctions of the zonulae adherens type; in muscle cells, they are represented by costameres, myotendinous junctions, and fasciae adherens.

Although the function of vinculin is unknown, the protein is essential for development of \textit{Caenorhabditis elegans} past the 2-fold stage and for movement of the body wall muscles (6). Recent observations of rat cardiocytes cultured on a silastic rubber substrate provide evidence that vinculin plays a role in assembly or function of transmembrane, force-transducing structures. Cardiocytes assemble myofilaments and beat rhythmically for several days before they acquire the ability to contract the silastic membrane. Development of this latter capacity is correlated with prominent localization of vinculin at each pleat in the silastic substrate (7). The morphology of these contact points, their relationship to the underlying myofilaments, and the presence of vinculin closely resembles the costamere structure of muscle in situ (8–10).

Vinculin is present in many cell types, including muscle where it is constitutively expressed. In contrast, \(meta\text{-}Vinculin\) has been found only in muscle cells (3, 12), where it is expressed in a developmentally regulated manner (13) and in platelets (11). The probable role of vinculin in transduction of force across cellular membranes suggests that \(meta\text{-}Vinculin\) may play a specialized role, perhaps even an essential role, in transcellular force transduction in muscle. For this reason we have initiated studies to describe structural and functional relationships between vinculin and \(meta\text{-}Vinculin\) (14).

Currently it is known that \(meta\text{-}Vinculin\) is antigenically similar to vinculin (2, 3) but larger on SDS1 gels. Vinculin has an electrophoretic mobility consistent with its calculated molecular mass from cDNA of 117 kDa (15, 16), whereas \(meta\text{-}Vinculin\) has an apparent molecular mass 17 kDa greater (3, 17). On isoelectric focusing gels, porcine and avian \(meta\text{-}Vinculin\) are more acidic than vinculin. In addition, chicken gizzard \(meta\text{-}Vinculin\) has different solubility properties than vinculin, and an 8–10-fold higher specific activity of endogenous Ser/Thr phosphorylation (17). The subcellular localization of vinculin and \(meta\text{-}Vinculin\), evaluated by microinjection of fluorescently labeled proteins (13) and by immunofluorescence localization with antibody specific to human \(meta\text{-}Vinculin\) (18), is coincident at 0.2-μm resolution. Vinculin (19, 21) and \(meta\text{-}Vinculin\) (17, 21) bind to talin, and vinculin binds to acidic phospholipids (22, 23) as does \(meta\text{-}Vinculin\).2

Definition of the structural differences between vinculin and \(meta\text{-}Vinculin\) will provide a basis for assessing their functional properties. Early studies addressing the relation-

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction.
2 C. DeBerry, unpublished data.
3 R. Johnson, unpublished data.
ship between the two proteins showed that they have distinguishable although nearly identical peptide maps (3, 17), are synthesized from separate mRNAs (3), and do not have a precursor-product relationship (17). Recently, Gimona et al. (24) isolated and sequenced a 68-residue meta-vinculin-specific peptide from porcine meta-vinculin. Based on sequence homology between residues at the termini of the porcine meta-vinculin-specific peptide with the sequence of chicken vinculin, the meta-vinculin peptide was positioned within the sequence of chicken vinculin between residues 912 and 913. Because the meta-vinculin peptide accounts for only 7.6 kDa of the apparent mass difference between vinculin and meta-vinculin, the question remained whether the meta-vinculin peptide represented the full extent of the difference between the two proteins.

The present study was undertaken to define the nature and location of the primary sequence differences between chicken vinculin and meta-vinculin and to establish the genetic basis for tissue-specific expression of meta-vinculin.

**EXPERIMENTAL PROCEDURES**

**Reagents**- [α-32P]dCTP, [γ-32P]ATP, and [α-32P]cTP were obtained from Du Pont-New England Nuclear. Restriction endonucleases were supplied by New England BioLabs and Bethesda Research Laboratories. Reverse transcriptase was obtained from Life Science Biotechnology (Tampa, Florida). RNase inhibitor was from Bethesda Research Laboratories. Thermus aquaticus (Taq) polymerase was obtained from Perkin-Elmer Cetus. Bluescript vectors were purchased from Stratagene (San Diego, CA); pCR1000 was from InVitrogen, Inc.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—** Vinculin and meta-vinculin were separated by SDS-gel electrophoresis (25) and transferred by electrophoresis (26) to a nitrocellulose membrane. Proteins were detected with an affinity-purified polyclonal antibody to P2, a synthetic peptide with the sequence (K)EFSLPSDIEDDYEP, which corresponds to a portion of the cDNA-derived chicken meta-vinculin-specific sequence.

**Isolation and Analysis of Vinculin Genomic Clones—** A chicken genomic library (gift of D. Engel, Department of Biochemistry, Northwestern University, Evanston, IL) was screened by hybridization with cDNA probes derived from pCE45V and pCE68V (27). Library western University, Evanston, IL) was screened by hybridization with purified by CsCl gradient centrifugation or by miniprep lysis (28). A library of genomic clones was isolated from a chicken erythrocyte genomic library obtained from Clonetech. Plaques were screened with a 150-bp EcoRI-Smal fragment of the meta-vinculin-specific region isolated from genomic clone Vgen-2 and one positive isolate, λ MV Q, was identified and plaque-purified. This clone is a partial cDNA which begins with an EcoRI site within the meta-vinculin exon followed by sequence coding for vinculin amino acids 915-1066, as well as 1.4 kb of the 3′-untranslated region. The 5′ portion of the meta-vinculin cDNA not represented in λ MV Q was isolated by amplification of reverse transcribed mRNA from 18-day embryonic gizzard (31). Oligo(dT) was used to prepare cDNA which was amplified in a PCR reaction with vinculin primers V12 (bp 2498-2522) and V13 (bp 2518-2546). The 3′-untranslated region of the meta-vinculin exon. Two products were obtained which differed by approximately 200 base pairs. Sequence analysis of the larger product confirmed this to be a cDNA which contained 207 base pairs of additional sequence coding for the additional 69 amino acids found in meta-vinculin. A meta-vinculin-specific primer (MV6, mv-bp 141-124) was used to generate a second pool of cDNA which served as a template for amplification of the remaining meta-vinculin cDNA. A second meta-vinculin-specific primer (MV9, w-bp 72-55) was used in combination with a vinculin primer V54 (bp 25-390) to amplify a 3-kb segment of the meta-vinculin cDNA. To obtain the final segment of the message, oligonucleotide V50 (bp 56 to 32 in the 5′-untranslated region (16)) was used in combination with V34 (bp 454-471) to generate the 5′ most fragment in a PCR reaction with the MV6-primed reverse transcriptase reaction products serving as template. These PCR products were sequenced following subcloning into pCR1000 (InVitrogen, Inc.) or were sequenced directly on an Applied Biosystems Inc. 373A DNA sequencing system.

**RESULTS AND DISCUSSION**

**Identification and Characterization of Genomic Clones for Vinculin—** A chicken erythrocyte genomic library in EMBL 3 was screened with a pool of vinculin cDNA clones representing bp 175 - 2645 of the 6.2-kb vinculin message (15, 27). Twenty-seven isolates were plaque-purified and characterized by hybridization with individual vinculin cDNA subfragments. Twenty of these clones hybridized to pCEV 3′-8-3 (bp 2649 -3227), which codes for vinculin amino acids 877-1066 (Fig. 1). These clones could contain the putative exon for a meta-vinculin-specific peptide analogous to the porcine meta-vinculin peptide that is known from peptide mapping studies to be present in this region (24). Therefore, the isolate with the largest genomic insert, Vgen-2, was chosen for analysis of exon/intron structure by restriction mapping and DNA sequencing.

**Determination of the Intron/Exon Structure of Genomic DNA Coding for the Carboxyl-terminal 370 Amino Acids of Vinculin—** Vgen-2, shown schematically in Fig. 2, is comprised of an 11.7-kb genomic Stall fragment and contains five consecutive EcoRI subfragments of 2.8, 0.4, 2.4, 0.6, and 5.5 kb. The 5′ end of the gene is joined to the left arm of EMBL 3. From nucleotide sequencing, we determined that amino acids 676-1066, found both in chicken embryo (15) and chicken fibroblast (16, 32) vinculin, are encoded by 9 exons ranging in size from 33 to 336 bp (Table I). The 5.5-kb EcoRI subfragment of Vgen-2 also encodes 1.4 kb of uninterrupted sequence which matches the 3′-untranslated region of the mRNA from vinculin (32) and meta-vinculin (this report).

![Fig. 1. Hybridization characteristics of vinculin-positive genomic isolates.](image-url)

Two million plaques from a chicken erythrocyte genomic library were screened with a pool of vinculin cDNA probes, corresponding to bp 175-2645 of the 6.2-kb vinculin message. Twenty-seven positive isolates were plaque-purified and hybridized with the probes indicated at the left of the schematic. Homology with a probe is indicated by +. Two distinct groups of clones are evident; those with homology to the sequences 5′ to the proline region, and those with homology to the vinculin tail domain which is in the carboxy-terminal one-third of the molecule. All isolates in the second group were positive for both vinculin and meta-vinculin coding region probes.
the 0.6-kb EcoRI fragment is the meta-vinculin specific probe; it
recognizes a 6.2-kb message. At the protein level, antibody raised
using a riboprobe generated from the meta-vinculin-specific
cDNA sequence has been submitted to GenBank with the accession
number M87837.

Identification and Characterization of the Exon Encoding
Chicken meta-Vinculin-specific Sequence—We observed that
amino acids 915 and 916 are encoded in separate exons, E896-
E915 and E916–E984, which are separated by 4 kb of inter-
vening sequence (Fig. 2; Table I). This exon structure would
permit generation of meta-vinculin by alternative splicing if
the intervening 4 kb contained a meta-vinculin specific exon(s). Analysis of the open reading frames in this region
revealed homology between the translated product of one open
reading frame (Fig. 3) and the 68-amino acid sequence specific
to porcine meta-vinculin (24). Direct evidence that this open
reading frame encodes sequences present in chicken meta-
vinculin mRNA was obtained by Northern blot analysis of
poly(A)+ RNA isolated from an 8-day embryo and adult gizzard
and fractionated on a 0.8% agarose-formaldehyde gel. Duplicate lanes
were run and blotted onto nitrocellulose. Riboprobes to the meta-
vinculin exon (EcoRI-SalI, 150 bp) or vinculin E896–E918 were used
to detect the 6.2-kb mRNA in both embryo and gizzard.

To determine the portion of the chicken meta-vinculin open
reading frame that is expressed in mRNA, a cDNA for meta-
vinculin was isolated (Fig. 6; see “Experimental Procedures”) and
sequenced. Of the 107 amino acids encoded by the ge-
nomic open reading frame (Fig. 3), 69 are present in meta-
vinculin cDNA (Fig. 3, boxed region of the amino acid se-
cquence). Of particular interest is a set of repeated peptides
(boxed segments, Fig. 7) which occur at the carboxy-terminal
end of the vinculin exon preceding the meta-vinculin insertion
and at the end of the meta-vinculin-specific sequence. These
repeats are an indication that a duplication event has occurred
with the vinculin exon E896–E915 in higher eukaryotes, since
these sequences are not represented in the del-1 gene of C.
elegans, which is homologous to vinculin (33).

Comparison of the Avian and Porcine meta-Vinculin-specific
Sequence—The homology between porcine and avian meta-
vinculin-specific peptides is greatest in the carboxy-terminal
portion where 37 of the last 40 amino acids are identical. In
the amino-terminal one-third, however, identity drops to 44%.
The chicken meta-vinculin-specific sequence is characterized by a calculated isoelectric point of 5.37. On isoelectric focusing gels, meta-vinculin from chicken gizzard has one prominent isofrom of pl 5.9 and a minor isoform of pl 6.2. Both of these are more acidic than the gizzard vinculin isofroms which range from pl 6.3 to 6.5 (34) (Fig. 8).

**Determination of the Complete meta-Vinculin Primary Structure from cDNA**—The apparent molecular mass difference between vinculin and meta-vinculin on SDS gels is 17 kDa. The 69-amino acid sequence specific to chicken meta-vinculin accounts for only 7.6 kDa of this mass difference. To determine whether there are additional sequence differences, a full-length cDNA for meta-vinculin was obtained by a PCR strategy (Fig. 7 and “Experimental Procedures”), subcloned, and sequenced with oligonucleotide primers. No other sequence differences between vinculin and meta-vinculin were found. Consequently, the 17-kDa shift in apparent molecular mass must in part reflect a conformational difference in meta-vinculin as compared with vinculin. In rotary shadowed images both vinculin and meta-vinculin show a globular head domain of 8 nm and an extended tail domain (35). The meta-vinculin tail region measures 21.5 versus 19.4 nm for vinculin (35); this difference may contribute to the slower mobility of meta-vinculin on SDS gels.

The existing data do not rule out the possibility that there are additional primary sequence isoforms of vinculin and meta-vinculin. The recent characterization of a cDNA having an in-frame deletion coding for amino acids 167–207, the putative talin-binding region, suggests that there may be

while the overall similarity is 72%. Three regions of the meta-vinculin-specific exon are discernible based on their degree of conservation and relationship to vinculin. The amino-terminal one-third is divergent between the porcine and avian peptides and unique to meta-vinculin. The carboxyl-terminal one-third is completely conserved between the two species and is similar to the carboxyl-terminal end of the preceding vinculin exon by a set of repeated sequences (Fig. 6). The middle third of the meta-vinculin segment is highly conserved and unique to meta-vinculin and is likely therefore to encode a specialized function for meta-vinculin.

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additional sequence isoforms (36). Also, the structural basis of the multiple isoelectric forms of vinculin and meta-vinculin (5, 34, 37, 38) remains to be established.

Evidence That a Single Gene Codes for Both Vinculin and meta-Vinculin—The second goal of this study was to determine whether vinculin and meta-vinculin are encoded in separate genes or are alternatively spliced products of a single gene. Clear information on this point is required prior to studies of the mechanism of muscle-specific expression of meta-vinculin. Earlier analyses of Southern blots probed with segments of vinculin cDNA produced variable results, sometimes identifying multiple bands (27, 32).

We have obtained three independent sets of data to address the genetic origin of meta-vinculin. The first evidence to suggest vinculin and meta-vinculin are encoded by a single gene is provided by the hybridization characteristics of 27 vinculin-positive isolates from the EMBL 3 library. All 20 of the isolates that contain sequence homologous to the tail domain of vinculin, detected by probe pCEV3 '83 (15), are also recognized by the meta-vinculin-specific probe (Fig. 1). These genetic data make it improbable that separate genes code for vinculin and meta-vinculin.

Second, unambiguous Southern blots were obtained using probes derived from sequence-defined exons lacking the restriction enzyme sites used to digest the genomic DNA (Fig. 8). Chicken genomic DNA was digested with SalI followed by a second digestion with either BamHI, PstI, PvuII, or EcoRI. A duplicate set of lanes was run on the same gel and then probed with either vinculin exon E916–E984, or the meta-vinculin-specific probe, an EcoRI-Smal fragment from Vgen-2 (Fig. 2). Single bands were detected in all four digests, indicating that a single gene is recognized by both probes. In the BamHI, PstI, and PvuII digests, both probes recognized the same size fragments, the sizes of which match those from corresponding digests of genomic clone Vgen-2 (data not shown). Results of the EcoRI digest demonstrate that the vinculin and meta-vinculin probes reside on separate fragments as predicted from the map in Fig. 2. The E916–E984 probe identifies a 5.5-kb EcoRI-SalI fragment, and the meta-vinculin probe identifies the smaller 0.6-kb EcoRI-EcoRI fragment. A restriction fragment length polymorphism in the 5.5-kb EcoRI-SalI fragment produced a doublet of 5.5 and 4 kb in several of 10 additional chicken genomic DNAs analyzed (data not shown). Heterozygotes at this locus may also have generated multiple bands in earlier experiments. This data strongly supports the presence of a single gene for both vinculin and meta-vinculin, since the coding segments physically map to the same DNA segment.

Last, the 1.4 kb of the 3'-untranslated sequence of meta-vinculin cDNA is identical to the 3'-untranslated sequence of vinculin cDNA (data not shown), and this sequence is encoded as a single segment in Vgen-2. In general, the 3'-untranslated portions of mRNA are not highly conserved between related genes, suggesting that vinculin and meta-vinculin derive from the same gene.

Together, these three independent sets of data establish that the tissue-specific expression of meta-vinculin occurs by alternative splicing of the mRNA product of a single gene that encodes the information for both vinculin and meta-vinculin; this gene is designated vinc. Based on the sequence identity of vinculin and meta-vinculin, we have summarized the structural and putative functional domains of meta-vinculin in Fig. 9. A key challenge for future experiments is to determine the role(s) that vinculin and meta-vinculin have in assembly or function of the transmembrane, force-transducing structures in muscle cells and other cell types.

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Sequence and Genetic Origin of Chicken meta-Vinculin