Porcine vinculin and metavinculin differ by a 68-residue insert located close to the carboxy-terminal part of the molecule

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Metavinculin is a higher mol. wt variant of vinculin expressed only in muscle tissue. Using amino acid sequencing methods on the intact molecules and their proteolytic subfragments, together with a polyclonal antibody specific only for metavinculin from porcine stomach, we have been able to identify and sequence the difference peptide in the porcine metavinculin molecule. By alignment with the complete sequence of chick fibroblast vinculin (communicated by G.J.Price, P.Jones, M.D.Davison, R.Bendori, S.Griffiths, B.Patel, B.Geiger and D.R.Critchley, prior to publication) the exact location of the insert could be determined. In porcine metavinculin, this insert lies between the 90-kD protease-resistant amino-terminal core and the carboxy terminus of the molecule. It contains 68 amino acids and is flanked by KWSSK sequences, one of which is present in vinculin. The identity of the mapped vinculin and metavinculin sequences outside this difference peptide is consistent with the two proteins arising via alternative splicing at the mRNA level. The lack of reactivity of the porcine metavinculin antibody with metavinculin from chicken as well as the finding of different proteolytic cleavage sites in avian metavinculin indicate a species-specific amino acid sequence in the difference piece of the metavinculin molecule.

Key words: vinculin/metavinculin/porcine/carboxy terminus/cleavage sites

Introduction

Vinculin is a ubiquitous, cytoskeletal-associated protein of 130 kD generally acknowledged as belonging to a group of components involved in actin–membrane linkage (Geiger, 1983; Mangeat and Burridge, 1984; Burridge, 1986). In muscle tissue, metavinculin, a structurally and immunologically related protein of 150 kD, is expressed together with vinculin (D'Angelo-Siliciano and Craig, 1982; Feramisco *et al.*, 1982). Metavinculin is particularly prevalent in smooth muscle (Saga *et al.*, 1985; Glukhova *et al.*, 1986) where it can occur in an excess over vinculin (Gimona *et al.*, 1987) and this tissue has therefore been the favoured source for its purification. Since both vinculin and metavinculin are produced by *in vitro* translation of smooth muscle mRNAs (Feramisco *et al.*, 1982) and both are

simultaneously labelled in *in vivo* pulse–chase experiments (D'Angelo-Siliciano and Craig, 1987) the possibility that metavinculin is a higher mol. wt precursor of vinculin has been excluded. Open questions remain about the structural differences between these molecules and in particular how they relate to the need for a higher mol. wt variant of vinculin in muscle tissue.

We have recently compared the biochemical and structural properties of avian and mammalian vinculins and metavinculins (Gimona *et al.*, 1987). As we and others (Feramisco *et al.*, 1982) have shown, limited proteolysis of both vinculin and metavinculin yields common, relatively protease-resistant core fragments of ~90 kD and a small number of characteristic low mol. wt peptides. By amino-terminal sequence analysis we show first that mammalian and avian vinculins and metavinculins as well as their core fragments share the same amino terminus. Further, using an antibody specifically directed against porcine metavinculin, we have been able to identify a proteolytic fragment unique to this variant of the molecule. Direct sequencing of this and other fragments and a comparison with the known cDNA sequence data for vinculin derived from chick fibroblasts (Price *et al.*, 1987; G.J.Price *et al.*, unpublished) has enabled us to determine the size, sequence and position of the metavinculin-specific peptide in the molecule. Taken together with additional sequence data on the avian and porcine molecules, the results strongly suggest that vinculin and metavinculin originate by differential mRNA splicing.

Results

Cross-species identity of amino-terminal sequence of the vinculin and metavinculin core fragments

Amino-terminal sequencing of the porcine and avian vinculins and metavinculins revealed complete identity for

Fig. 1. Sequence identity of the 90-kD core fragments of porcine vinculin and metavinculin with chick fibroblast vinculin. Amino acid sequence analysis of the intact core and its 67-kD and 39-kD subfragments showed exact alignment with the chick fibroblast vinculin sequence in positions 1–20, 244–260 and 495–511. Cleavage sites at positions 243 and 494 are consistent with a three-domain structure in the core. By comparison with the chick vinculin sequence (Price *et al.*, 1987), the 90-kD V8 core is located between residues 1 and 857.

Fig. 2. Characterization of metavinculin-specific antibody. (A,A') SDS gel of hog stomach whole muscle (A) and corresponding immunoblot stained with metavinculin-specific antibody, showing reaction with metavinculin only (A'). (M,B,C) SDS gel of the samples used for the adjacent immunoblots; M, marker lane including vinculin and metavinculin (for other bands see below); B, hog stomach vinculin; C, hog stomach metavinculin. (B',C') Immunoblot of samples in (B) and (C) with metavinculin-specific antibody showing reaction with only intact metavinculin. (B'',C'') Immunoblot of samples in (B) and (C) with a monoclonal anti-vinculin antibody that recognizes vinculin and metavinculin. M, mol. wt standards (200, myosin heavy chain; 150, metavinculin; 130, vinculin; 92.5, phosphorylase A; 68, BSA; 57, pyruvate kinase; 51, hexokinase; 42, actin; 30.6, carbonic anhydrase; 20, myosin light chain; 12.5 kd cytochrome *c* from top to bottom). (D–F, D'–F') Specificity of metavinculin antibody for the 30-kd fragment. SDS gel and corresponding immunoblot of metavinculin fragments of 30-kd chymotrypsin (lanes D and D'), 24-kd papain (lanes E and E') and 20-kd V8 (lanes F and F'). Note specific labelling is only obtained on the 30-kd chymotryptic fragment; all other fragments are negative. D–F Coomassie blue gel; D'–F', corresponding immunoblot. Numbers at the sides indicate mol. wts in kd.

at least the first 20 amino acids (Figure 1). The same amino terminus was also identified in all large sized core fragments obtained by limited proteolysis, definitively siting these fragments at the amino-terminal end of the molecules. This directly confirmed earlier assumptions about the location of the core fragment (Jones *et al.*, 1987).

Using a monoclonal antibody that reacted specifically with the 90-kd core piece (see following section), breakdown products from the core fragment obtained by extended enzymatic cleavage with V8 protease could be identified and partially sequenced from the glass fibre electroblots. Alignment of the main core subfragments (67 kd and 39 kd) with the known chick fibroblast vinculin sequence showed remarkable identity within the cores of the avian and mammalian molecules (Figure 1).

Further evidence for cross-variant and cross-species homology within the 90-kd fragments was the identity of the peptide separation patterns on SDS gels generated by chemical cleavage of HPLC-purified core fragments in 80% formic acid (data not shown).

A metavinculin-specific antibody recognizes an epitope C-terminal to the 90-kd core fragment

Adsorption of an affinity-purified pig metavinculin polyclonal antibody on an affinity column of vinculin yielded an antibody fraction that specifically labelled metavinculin and not vinculin in samples of porcine stomach smooth muscle (Figure 2A,A'). In cross-sections of porcine stomach muscle the metavinculin-specific antibody showed the same cell

Fig. 3. Cross-section of hog stomach smooth muscle stained with metavinculin antibody. (Bar = 10 μm).

border label (Figure 3) characteristic for antibodies that do not discriminate between metavinculin and vinculin. The immunoblots shown in Figure 2 compare the reaction of the metavinculin-specific antibody (B' and C') with that of the non-discriminating monoclonal antibody (B'' and C'') on

Fig. 4. Purification of metavinculin and vinculin fragments by reversed-phase HPLC. (A) V8 digest of metavinculin with accompanying gel (inset) of the two major peaks: lane 1, 14–16-kd doublet; lane 2, 20-kd fragment; lane 3, 90-kd core fragment. (B) Papain digest. Gel (inset): lane 1, 24-kd fragment; lane 2, 100-kd fragment. (C) Chymotrypsin digest. Gel (inset): lane 1, 30-kd fragment; lane 2, 105- and 90-kd fragments. (D) Vinculin V8 digest. Gel (inset): lane 1, 26–28-kd doublet; lane 2, 90-kd fragment.

samples of porcine vinculin (B) and metavinculin (C). As is evident from Figure 2B' and C' the metavinculin antibody failed to recognize vinculin. A similar analysis on the metavinculin large sized core fragments also failed to reveal any cross reactivity (Figure 2D'–F'). In view of the location of the 90-kd fragment (see above) it could be concluded that this antibody was directed against a carboxy-terminal part of the molecule situated beyond the 90-kd core fragment.

As we have shown previously (Gimona *et al.*, 1987), limited proteolysis of vinculin and metavinculin yields characteristic low mol. wt fragments. The HPLC separation patterns and the corresponding electrophoresis gels of the fragments of porcine vinculin and metavinculin are shown collectively in Figure 4. Digestion of metavinculin with the V8 protease yields the 90-kd fragment in addition to a 20-kd peptide and a doublet at 14–16 kd (Figure 4A). Papain cleaves the protein to yield an intermediate 100-kd fragment and a 24-kd polypeptide (Figure 4B). Finally, chymotrypsin digestion produces fragments of 105 kd and 30 kd (Figure 4C). More extensive chymotryptic degradation leads to the loss of the 30-kd peptide and the concomitant appearance of a 16-kd piece. This latter peptide was most easily obtained by gel electrophoresis followed by electroblotting (data not shown). Of these low mol. wt fragments the porcine

metavinculin-specific antibody reacted strongly only with the metavinculin fragment of 30 kd and not with the 24-kd and 20-kd peptides (Figure 2). The 16-kd fragment of the chymotryptic digest was also not recognized by the antibody and, as we show below, constitutes the carboxy-terminal piece common to both vinculin and metavinculin. As will become evident from the alignment of these fragments (see below and Figure 6), the porcine metavinculin-specific epitope resides in the amino-terminal part of the 30-kd chymotryptic fragment.

The porcine metavinculin-specific antibody reacted only poorly and non-specifically with avian metavinculin. Furthermore the cleavage patterns for avian metavinculin looked somewhat different from those obtained with porcine stomach metavinculin, suggesting a different primary structure for the difference piece in the avian molecule (data not shown).

Porcine metavinculin contains an extra 68-amino-acid insert between the core and the carboxy-terminal domain

As shown in Figure 5, the amino-terminal sequences of the 30-kd, 24-kd, 20-kd and 16-kd fragments obtained from

Fig. 5. Comparison of the amino acid sequences derived for porcine vinculin and metavinculin with part of the cDNA translated sequence obtained for chicken fibroblast vinculin (G.J.Price *et al.*, unpublished). The amino acid sequences flanking and covering the difference peptide typical of porcine metavinculin (m-Vin) are shown. Comparison is made with the corresponding region of porcine vinculin (Vin). The part absent in vinculin is shown by a dashed line. Numbers indicate amino acid positions in the translated cDNA sequence of chick fibroblast vinculin (G.J.Price *et al.*, unpublished). Amino acid exchanges with the avian protein are shown in the upper row (positions 851, 853, 892 and 893). Small stars indicate deletions in positions 901 and 902. Vertical arrows indicate the cleavage sites by the V8 protease (V8), chymotrypsin (Ch) and papain (Pap). Horizontal arrows delineate the beginnings of the sequences covered in this study. The positions of the tryptic peptides are also shown (T). Only those peptides necessary to document the given sequence are shown; overlapping peptides are omitted. Boxed residues show an identical repeat in the amino-terminal flanking region and the metavinculin insert.

metavinculin overlapped each other in that order, to give a stretch of 92 residues. This accumulated sequence aligned, after 68 positions, with the C-terminal sequence of the chick fibroblast vinculin. The latter C-terminal sequence began at the cleavage site of the chymotryptic 16-kd fragment.

The N-terminal sequence of the mixture of the 14–16-kd V8 fragments could be fitted into the chick fibroblast vinculin sequence (Price *et al.*, 1987) starting at positions 851 and 858 respectively. The complete sequences of these two fragments were derived from overlapping tryptic and V8 protease peptides and extended through a part of the proline-rich stretch of the molecule (see Figure 5) and into the amino-terminal region of the 30-kd chymotryptic and 24-kd papain fragment of metavinculin terminating at the V8 20-kd cleavage site. These sequences then defined the amino-terminal flanking region of the metavinculin-specific insert.

When porcine vinculin was treated with V8 protease under non-denaturing conditions, a 26–28-kd doublet (Figure 4D) was obtained that revealed a similar double sequence to that observed for the 14–16-kd doublet of metavinculin (see above). Using overlapping sequences from tryptic and V8 protease peptides, the amino-terminal sequence of this 26–28-kd doublet could be extended over 85 amino acids (Figure 5). This sequence was identical to that deduced for the metavinculin stretch except that it lacked an insert of 68 amino acids. Thus the metavinculin-specific insert could be located between positions 912 and 913 in the vinculin sequence (assuming correspondence with the avian positions).

It was noteworthy that the 68-amino-acid insert not only started but also terminated with the same KWSSK motif. Furthermore it contained a pattern of amino acids constituting a repeat of a region located in the amino-terminal flanking region common to both vinculin and metavinculin (boxed in Figure 5).

Fig. 6. Schematic model of mammalian vinculin (top) and metavinculin (bottom). Domains common to both proteins are indicated: open bar, 90-kd core fragment; dotted area, proline-rich linking region between 90-kd core and the beginning of the insert (for metavinculin) and the C-terminal fragment (for vinculin) respectively; hatched area, C-terminal fragment. In metavinculin the solid area marks the location of the metavinculin-specific insert which carries two additional cleavage sites for V8 protease and papain (see enlarged space). Symbols: papain (■), chymotrypsin (▼) and V8 (●) cleavage sites. N and C denote the amino- and carboxy-terminal sides of the molecule.

We also identified four amino acid exchanges between the chick fibroblast vinculin and the porcine vinculin/metavinculin sequence in the flanking regions. They are located at positions 851 (H → Q), 853 (H → R), 892 (G → E) and 992 (A → V) (see Figure 5). The amino acid substitution at the site 851 might be responsible for the alternative cleavage by the V8 enzyme in this region (positions 850/851 and 856/857) yielding the doublet fragments 14–16 kd (for metavinculin) and 26–28 kd (for vinculin). Indeed, similar digestion conditions used with turkey gizzard vinculin produced full cleavage between residues 856/857 yielding only a single peptide of ~26 kd (results not shown).

Finally and surprisingly, we observed an apparent deletion of two alanine residues in the mammalian vinculin and metavinculin molecules as compared to chick fibroblast vinculin, in positions 901 and 902 (small stars in Figure 5).

Discussion

The sequence data obtained here, taken together with the extended cDNA sequence derived from chick fibroblast vinculin (Price *et al.*, 1987; G.J.Price *et al.*, unpublished) allows us to present a complete model of porcine metavinculin (Figure 6). As we have shown, the 90-kd core fragment constitutes the amino-terminal part of the molecule, is probably identical in primary structure in vinculin and metavinculin and is remarkably conserved between species. The core domain itself contains two major protease sensitive sites as revealed by the formation of 67-kd and 39-kd fragments upon extensive digestion with V8 protease. The presence of these sites is consistent with a three-domain organization of the core and with the 3-fold repeat previously deduced by Price *et al.* (1987) from the cDNA-derived sequence. The 90-kd domain is linked at its carboxy-terminal side to a proline-rich sequence extending over ~40 residues. Since proline residues tend to favour *cis*-peptide conformations, this linkage region might represent a special structural domain. Interestingly this section is bisected by a V8 protease-sensitive bond. In keeping with the identity of the 90-kd domains, no amino acid differences could be noted between the deduced vinculin and metavinculin sequences in this linkage region. Furthermore, and finally, it could be concluded from comparative peptide and partial sequence analysis that the 16-kd piece was located at the carboxy terminus of both vinculin and metavinculin. Comparison of the sequence of the common regions of porcine vinculin and metavinculin with that derived from chicken vinculin cDNA clone also revealed a very high conservation of the primary structure. So far we have identified only four exchanges and two deletions in regions covering at least 154 residues (see Figures 1, 5 and 6).

The difference peptide of metavinculin is inserted between the carboxy terminus and a site some 50 amino acids beyond the end of the 90-kd core. The insertion in pig metavinculin is 68 amino acids long and contains additional sites sensitive to V8 protease and papain. This fortuitous pattern of cleavage, yielding overlapping fragments, facilitated the assignment of the primary structure of the insertion by direct amino acid sequence strategies. A notable feature of the insertion sequence is the presence of identical KWSSK flanking sequences. These motifs probably possess a similar secondary structure as both exhibit chymotrypsin-sensitive sites. Indeed, from secondary structure predictions (Chou and Fasman, 1978; Garnier *et al.*, 1978) it follows that the KWSSK sequences are in close proximity to or part of β -turns each flanked outside the metavinculin insert by stable, long α -helices (residues 879–910 and residues 919–937). The insert itself has less pronounced structure and most likely consists of two α -helices (about eight residues) interspaced by three coil regions. Such an organization could easily represent an independent structural domain in the molecule. D'Angelo-Siliciano and Craig (1987) reported an increased number of phosphorylation sites in avian metavinculin as compared to vinculin. We have likewise identified potential phosphorylation sites in the porcine molecule, some con-

taining serine and a single site with a consensus substrate sequence of a tyrosine kinase (Hunter and Cooper, 1985; De *et al.*, 1986).

It was of interest to find that metavinculin antibodies did not react convincingly with avian metavinculin. Neither did the gizzard metavinculin yield lower mol. wt fragments equivalent to those obtained from the porcine molecule. Therefore, although we expect a similar extra peptide to exist in avian metavinculin, this must be sufficiently different to explain the lack of cross-species identity. From the absence of cross-reactivity with the other small mol. wt fragments, the porcine metavinculin-specific epitope could be situated in the 26 amino-terminal residues of the insert. The very obvious flanking of the metavinculin insert by a duplication of the KWSSK sequence, the probably complete identity between vinculin and metavinculin in the rest of the molecule and the findings of other groups (Price *et al.*, 1987; Bendori *et al.*, 1987) that there might be only one large mRNA lead us to conclude that these two proteins are derived from one single gene via alternative splicing at the mRNA level. A site of mRNA splicing is tentatively located in the chick fibroblast cDNA sequence (G.J.Price *et al.*, unpublished) at the TG/GT sequence encoding the Trp-Ser residues at positions 911–913 (Figure 5) in line with the proposed consensus sequence at splicing junctions (A)UG/GU–intron–AG/GU (see Kourilsky and Chambon, 1978; Crick, 1979). A definitive assignment of the splicing position must await analysis of the corresponding pig genomic DNA sequence.

The size of the difference peptide (68 amino acids) and of the vinculin molecule (estimated from cDNA sequence) indicate that the real mol. wts of vinculin and metavinculin are far removed from those estimated by SDS gel electrophoresis. From the collected sequence data (G.J.Price *et al.*, unpublished) the respective mol. wts may be estimated as 116.5 kd for vinculin and 123.9 kd for metavinculin. The origin of the isoelectric variants found for both vinculin and metavinculin remains so far unexplained, but it is not unlikely that they arise by different degrees of post-translational modification.

Apart from the demonstrated specificity of metavinculin for smooth and cardiac muscle (Feramisio *et al.*, 1982; D'Angelo-Siliciano and Craig, 1977; Gimona *et al.*, 1987; Glukhova *et al.*, 1986) any functional differences between vinculin and metavinculin have so far escaped detection. With the complete sequence data now available as well as the proteolytic fragments derived from different parts of the molecule, tools are now at hand to search for molecular partners that may define differentiation linked tissue specific interactions that must occur.

Materials and methods

Proteins and protein cleavages

Vinculin and metavinculin from pig stomach and chicken and turkey gizzard were purified according to Gimona *et al.* (1987). The proteins were used fresh or were lyophilized for storage after dialysis against 0.02% sucrose.

Digestion with α -chymotrypsin, papain and *Staphylococcus aureus* V8 protease (Sigma) was performed at room temperature under non-denaturing conditions (20 mM KCl, 20 mM imidazole, 0.5 mM EGTA, pH 7.3) or under denaturing conditions in a buffer containing 1% SDS, 2 mM EDTA and 250 mM Tris-base, pH 6.8. Samples were processed for electrophoresis as below. The 90-kd core fragments were additionally treated with trypsin or V8 protease (at an enzyme to protein ratio of 1:50 for 3 or 20 h respectively) and the peptides separated by reversed-phase HPLC (see

below). Peptide cleavage was also performed under conditions known to be specific for Asp-Pro bonds. For this, HPLC-purified fragments were treated in 80% formic acid at 37°C for 24 h. The fragments were separated by SDS-acrylamide gel electrophoresis and compared after silver staining according to the procedure of Tunon and Johansson (1984).

Peptide separation and amino acid sequencing

Analytical gel electrophoresis was carried out on 8–22% acrylamide minislab gels according to the procedure of Matsudaira and Burgess (1978) in the buffer system of Laemmli (1970). Protein samples were supplemented with SDS sample buffer (final concentrations: 2.5% SDS, 1% 2-mercaptoethanol, 7% glycerol, 0.001% bromophenol blue, 62.2 mM Tris, pH 6.8) and boiled for 1 min at 100°C. The gels were stained for 30 min at 60°C in 0.2% Coomassie brilliant blue G250 (Sigma) dissolved in 50% methanol and 10% acetic acid and destained in 10% acetic acid at 70°C.

Gel separations used for protein electroblotting and sequencing were carried out on 1.5-mm-thick slab gels using 20% polyacrylamide concentrations. The separated proteins or fragments were electroblotted onto glass fibre sheets which were coated with poly(4-vinyl-N-methylpyridine). The glass fibre membrane-immobilized proteins were detected by reaction with diluted fluorescamine in acetone (1 mg/ml) and visualized under UV light. The membrane surface containing the proteins was cut out and mounted in the reaction chamber of an Applied Biosystems Inc. 740 A gas phase sequencer equipped with an on-line 120 A phenylthiohydantoin amino acid analyser. Both sequencer and analyser were operated as described by the manufacturer. Details on the blotting procedure are given by Vandekerckhove *et al.* (1987) and Bauw *et al.* (1987).

Alternatively, fragments of digested vinculin and metavinculin, obtained by either limited or complete digestion, were separated by reversed-phase HPLC using a 0.46 × 25 cm column (VYDAC, Separations Group, USA). Peptides were eluted with a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid applied with a slope of 1%/min. Peptides were detected by absorption at 215 nm, collected by hand and dried in a Speed Vac (Savant Instruments). Aliquots were used for further enzymatic or chemical cleavage or for direct sequence analysis (see above).

Immunological techniques

Polyclonal antisera were raised in two rabbits against pig stomach metavinculin according to the procedure described by Demey (1983). The metavinculin-specific antibody was purified from a polyclonal serum by sequential chromatography on affinity columns of pig stomach vinculin and metavinculin obtained by coupling purified vinculin or metavinculin to CNBr-activated Sepharose 4-B (Pharmacia Fine Chemicals, Sweden).

A monoclonal antibody to vinculin (antibody vin 11/5, Sigma) was a gift from Professor B.Geiger (The Weizmann Institute, Rehovot, Israel).

Blotting of minislabs onto nitrocellulose sheets (Schleicher and Schuell, FRG) was performed according to Towbin *et al.* (1979) and silver-enhanced immunogold staining carried out as described by Moeremans *et al.* (1984) using a secondary antibody with a 20-nm gold tag (Janssen Pharmaceutica, Belgium).

Immunofluorescence microscopy was performed on ultrathin sections of hog stomach smooth muscle embedded in polyvinylalcohol (Small *et al.*, 1986). Fixation was in a mixture of 0.3% Triton X-100 and 3% paraformaldehyde for 30 min.

Sections were observed and photographed in a Zeiss photomicroscope III equipped with epifluorescence optics.

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