Variable and constant regions in the C-terminus of vinculin and metavinculin

Cloning and expression of fragments in E. coli

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Metavinculin differs from vinculin in having an additional insert of 68 to 79 amino acids in length in the C-terminal half of the molecule. Cross-species comparison of metavinculin sequences from pig, man, chicken and frog reveals a division of the insert into two parts: the first variable and the second highly conserved. The longest insert, 79 amino acids, was found in Xenopus laevis. Three different C-terminal constructs of vinculin and metavinculin over-expressed in E. coli could be purified by column chromatography. Two-dimensional gel electrophoresis and peptide analysis revealed pI values between 8.35 and 10.75 for the recombinant proteins. Biochemical and structural features of the metavinculin-specific sequence and the conserved vinculin/metavinculin carboxy-terminus are discussed.

1. INTRODUCTION

Anchorage of the actin cytoskeleton to the cell membrane in cell contact areas is mediated by a cascade of interacting cytoplasmic and transmembrane proteins [1-3]. The biochemical composition of this complex shows some tissue variability and the number of newly identified proteins associated with it is rapidly increasing [4-6]. One protein ubiquitously found in actin-associated cell-matrix and cell-cell contacts is the cytoplasmic 117 kDa protein vinculin [7]. In smooth muscle cells an additional 124 kDa form, named metavinculin, is expressed [8,9]. Recently it has been shown that vinculin and metavinculin arise from a single gene by alternative splicing of a single exon coding for a 68 (mammalian) or 69 (avian) amino acid stretch inserted in the carboxy-terminal third of the molecule [10,11]. Structure-function analysis revealed that vinculin contains several binding sites involved in its association with cell adhesions [12]. Jones et al. [13] showed that the amino-terminal region of vinculin contains a binding site for talin [14], another member of the anchorage cascade. Recently Turner and colleagues [4] showed that paxillin, a 68 kDa focal contact protein, binds in vitro to the C-terminal 28 kDa portion of vinculin. Moreover, constructs comprising either the N- or the C-terminal regions of the molecule that were introduced into cells via transfection with partial cDNAs localized specifically to focal contacts [15]. The functional aspects of the metavinculin-specific sequence is, however, still unclear. The aim of the present work was to compare the metavinculin specific sequences from a variety of different species in an attempt to identify common structural motifs. In addition, constructs of different portions of the vinculin/metavinculin molecule have been expressed in bacteria and purified by column chromatography. The finding that the loss of the C-terminal half of vinculin leads to unstable constructions points to a possible role of this region in stabilizing the protein.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and enzymes

E. coli DH 5α (GIBCO BRL, USA) was used as a standard host and the plasmid pBluescript (Stratagene) as a standard vector for cloning and sequencing of DNA fragments. The plasmid pMW 172 [16] and E. coli strain BL21 (DE3) [17] were a kind gift from Dr. Michael Way (MRC Cambridge, UK). All enzymes used were purchased from Boehringer Mannheim and New England Biolabs and used according to the manufacturer's recommendations.

2.2. Cloning, sequencing and expression of metavinculin-specific fragments

RNA was isolated from porcine stomach and chicken gizzard smooth muscle according to Chirgwin et al. [18] and subsequently
Fig. 1. Cross-species comparison of the C-terminal region of the metavinculin molecule including the metavinculin-specific sequences reveals high homology (boxed) throughout the C-terminus outside the metavinculin-specific piece. The metavinculin-specific sequences show a striking separation into a variable and a conserved domain (open and boxed regions). The grey areas indicate the KWSSK motifs flanking the metavinculin inserts. Sequences for chicken and human vinculin and metavinculin were taken from [10, 11, 21, 29].

Fig. 2. Comparison of secondary structure predictions according to Garnier-Osguthorpe-Robson analysis of the metavinculin inserts reveals structural similarity in the conserved region (con.) but divergence in the variable portion (var.). a, porcine; b, human; c, chicken and d, *Xenopus*.
3. RESULTS AND DISCUSSION

3.1. Inter-species comparison of vinculin and metavinculin sequences

Koteliansky et al. [10] and Byrne et al. [11] have shown that metavinculin and vinculin arise by alternative splicing of a single exon encoding the metavinculin-specific insert in chicken and human. The splice sites were identified at amino acid positions 915-916 of the vinculin sequence. The data shown here for the porcine and the *Xenopus* cDNAs are in agreement with these findings. For both of the latter species the nucleotide sequences encoding the single KWSSK motif of vinculin and the first upstream KWSSK motif in metavinculin
Table I
Calculated molecular weights and isoelectric point values of expressed fragments

<table>
<thead>
<tr>
<th>Construct</th>
<th>Mol. mass (Da)</th>
<th>Isoelectric point (pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW-2</td>
<td>28,822</td>
<td>8.35</td>
</tr>
<tr>
<td>MW-13</td>
<td>21,755</td>
<td>10.25</td>
</tr>
<tr>
<td>MW-13c</td>
<td>12,795</td>
<td>9.35</td>
</tr>
</tbody>
</table>

Note the extremely basic value of the MW-13 construct, representing the complete vinculin C-terminus.

were identical, whereas sequences coding for the second downstream motif in metavinculin were different (not shown). Furthermore, comparison of the porcine vinculin and metavinculin PCR clones showed nucleotide sequence identity outside the insert region (not shown). Taken together, these data suggest that the splice site is conserved in all tissues expressing metavinculin.

Species comparisons of vinculin sequences, C-terminal to the vinculin/metavinculin splice site revealed complete identity apart from three amino acid exchanges, one of them being conservative (R→K) at position ‘132’ in the *Xenopus* sequence (Fig. 1). The cDNA fragments coding for the metavinculin-specific insert piece from porcine and avian smooth muscle tissue were described in a previous part [10]. The metavinculin C-termini including the metavinculin-specific fragment from *Xenopus laevis* and porcine smooth muscle were obtained by PCR as described in section 2. Cross-species comparison of the metavinculin insert regions of the four species investigated (Fig. 1) show that the respective region from *Xenopus laevis* is longer (79 amino acids) than that from chicken (69 amino acids) or those from human and pig (68 amino acids). Furthermore, alignment of the peptide sequences reveals a variable sequence stretch in the N-terminal portion of the insert, but strong similarity throughout the C-terminal half. The demonstrated tissue-specific expression of metavinculin [9,18,27] together with the observed variability in the amounts of metavinculin expressed in smooth muscle tissue of different origin point to a distinct function of this vinculin variant.

3.2. Structural and physicochemical analysis of the insert and C-terminal sequences

Structural analysis of the four metavinculin insert sequences obtained by applying the Garnier-Osguthorp-Robson algorithms showed a common, conserved C-terminal half and a variable N-terminal region. As suggested earlier [19], the insert is predicted to contain 3 helices interspersed by B-turns (Fig. 2).

The pI values calculated for the short metavinculin-specific peptides demonstrate a strong acidic nature with values ranging from 3.86 (chicken) to 4.21 (human). In contrast, the C-terminus of vinculin is seen to be extremely basic (pI 10.6) and has a predicted high helical content (>52%).

3.3. Expression of constructs in E. coli

To provide tools for probing for specific functions of the metavinculin insert piece we have constructed several vinculin- and metavinculin C-terminal fragments.
Construct MW-2 spans the region from the beginning of the metavinculin insert throughout the complete C-terminus. MW-13 comprises the C terminus lacking the metavinculin difference piece and MW-13c encodes a C-terminal peptide starting at the StuI restriction site. These three constructs were over-expressed in *E. coli* and could be purified by column chromatography (Fig. 4). The calculated, basic isoelectric points (see Fig. 5. Two-dimensional nonequilibrium electrophoresis gels of expressed constructs. (A) MW-2; (B) MW-13; (C) MW-13c. The mobility is consistent with their predicted pI values (indicated).
Table I) are consistent with the observed migration of these fragments on two-dimensional nonequilibrium electrophoresis gels (Fig. 5).

While we were able to express the entire C-terminal domain of vinculin we failed, however, to express the metavinculin-specific insert alone in E. coli. Furthermore, the insertion of a stop codon at the StuI site (constructs MW-2s and MW-13s) did not yield the corresponding peptides in E. coli. We therefore conclude that C-terminal sequences play a crucial role in stabilizing the mRNA or the expressed protein. As described earlier [15], the C terminus of vinculin contains a region that can bind autonomously to focal adhesions. This site might overlap with the binding site for paxillin [4]. It is intended to use the purified constructs described in this communication, covering different lengths of metavinculin and vinculin, to define the binding motifs of these proteins in more detail as well as to identify as yet unknown binding partners.

It is noteworthy that the vinculin C-terminal sequences downstream of the KWSSK motif show 98.7% homology even between widely separated vertebrate species. In contrast, the KWSSK motif occurs as QWSSQ in nematode vinculin [28] and the nematode/vertebrate sequence homology for the C-terminus is only 68% [19] indicative of a branch point in the evolution of these molecules.

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REFERENCES