

# Properties of Smooth Muscle Meta-Vinculin

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**Abstract.** Quantitative studies show that meta-vinculin is ninefold more soluble in 0.6 M salt than in the 0.01 M salt buffers used to extract vinculin. Based on this finding, we have developed a protocol for the purification of meta-vinculin in 43% yield and 98% purity from a high salt extract of gizzard smooth muscle. In contrast to our earlier extraction studies, which were done on unfixed cryostat sections (30), the present studies done on tissue homogenates show that nonionic detergents are not required for solubilization of meta-vinculin. Furthermore, neither purified nor partially purified meta-vinculin binds to Triton X-114 micelles. Purified meta-vinculin is a monomeric, asymmetric molecule with a Stokes radius of 50.9 Å, a sedimentation coefficient of 6.35S, and a frictional ratio of 1.46. The calculated molecular weight of meta-vinculin is 145,000. Meta-vinculin has two isoforms of pI 5.9 and 6.2, and is phosphorylated *in vivo* to eightfold greater specific activity than vinculin. On immunoblots of

smooth muscle proteins, [<sup>125</sup>I]meta-vinculin binds specifically to talin and also to unidentified polypeptides of 180, 150, 95, 70, 68, and 45 kD. On two-dimensional peptide maps, iodinated vinculin and meta-vinculin have at least 95% of their major chymotryptic peptides in common, but each protein also has at least one highly labeled peptide that appears to be unique. Comparative peptide maps of high salt soluble meta-vinculin and the low salt soluble 152-kD protein (described by Feramisco, J. R., J. E. Smart, K. Burrige, D. Helfman, and G. P. Thomas, 1982, *J. Biol. Chem.*, 257:11024–11031) demonstrate extensive similarities among the vinculin-like proteins but suggest a lack of complete identity. *In vivo* pulse-chase experiments show that meta-vinculin and vinculin do not have a precursor-product relationship. The biochemical and structural differences found between vinculin and meta-vinculin suggest that there is a unique function for meta-vinculin in smooth muscle.

Two groups of investigators have described a 150–152-kD protein that is antigenically (15, 30) and structurally related to vinculin, the 130-kD protein found at a variety of cellular adhesive structures (3, 8, 16, 18, 19, 26, 27, 32). Comparison of these molecules with vinculin by two-dimensional peptide mapping (29, 31), or by one-dimensional mapping on reverse phase columns (15), demonstrates that the 150–152-kD molecule(s) are very homologous to vinculin. However, *in vitro* translation of gizzard mRNA indicates that the 152-kD protein and vinculin are synthesized from distinct mRNAs (15).

Whereas the 152-kD protein described by Feramisco et al. (15) was isolated from the same low ionic strength extract of gizzard that is used for the purification of vinculin (14), we discovered a similar protein, meta-vinculin, precisely because it was extracted poorly in this low ionic strength buffer (30). Therefore, either there are two 150-kD vinculin-like proteins with distinct solubility properties or there is a single protein that is sparingly soluble in buffers of low ionic strength.

Little is known about these large, vinculin-related proteins except that their expression is restricted to muscle cells and possibly to smooth muscle cells (15, 30), whereas vinculin is expressed in many cell types (3, 8, 16, 18, 19, 26, 27, 32).

To facilitate further study of meta-vinculin, we have developed a high yield purification protocol. Using the purified protein we have determined several physical and biochemical characteristics of meta-vinculin, established its relationship to the low salt soluble 152-kD protein, and identified several meta-vinculin binding proteins in smooth muscle.

## Materials and Methods

### Purification of Meta-Vinculin from Gizzard Smooth Muscle

The protease inhibitors used in the purification of meta-vinculin were protease inhibitor cocktail I (PIC I;<sup>1</sup> leupeptin, 1 mg/ml [Peninsula Laboratories, Inc., Belmont, CA]; antipain, 2 mg/ml [Peninsula Laboratories, Inc.]; trasyolol, 10 U/ml [Moby Chemical Co., Pittsburgh, PA]; benzamidine, 10 mg/ml [Sigma Chemical Co., St. Louis, MO]), and protease inhibitor cocktail II (chymostatin, 1 mg/ml; pepstatin, 1 mg/ml. Both inhibitors were purchased from Peninsula Laboratories, Inc., and dissolved together in DMSO.)

1. *Abbreviations used in this paper:* PIC I, protease inhibitor cocktail I (leupeptin, 1 mg/ml; antipain, 2 mg/ml; trasyolol 10 U/ml; benzamidine, 10 mg/ml); PIC II, protease inhibitor cocktail II (chymostatin, 1 mg/ml; pepstatin, 1 mg/ml); HAP buffer, hydroxylapatite column buffer (10 mM phosphate buffer; 0.15 M NaCl; 0.02% NaN<sub>3</sub>; pH 7.3).

All Tris-containing buffers were pH'd at room temperature and used at 4°C for DE-52 chromatography or at room temperature for HPLC chromatography.

200 g of glycerinated smooth muscle from gizzards of adult chickens was rinsed in distilled water and then homogenized in 1,000 ml of 0.6 M Tris-HCl, 1 mM EGTA, 1 mM EDTA, 10 U/ml trasyolol, 1 ml PIC I, and 1 ml PIC II, pH 7.4, at 4°C with three 10-s pulses in a Waring blender operated full speed. The homogenate was stirred for 30 min at 4°C and then centrifuged for 30 min at 12,000 g. The pellet was discarded. The supernatant was dialyzed against 2 × 10 liter changes of 50 mM Tris, pH 7.4, 10 U/ml trasyolol for 24 h at 4°C. The dialyzed extract was centrifuged at 12,000 g for 30 min. Ammonium sulfate was added to the supernatant to bring it to 17% saturation. The precipitated protein was removed by centrifugation and the pellet was discarded. The supernatant was brought to 30% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitated material, which contains meta-vinculin, was collected by 12,000 g centrifugation. This pellet was dissolved in DE-52 column buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 10 U/ml trasyolol, 0.02% NaN<sub>3</sub>, pH 7.4), dialyzed for 24 h vs. DE-52 column buffer, and then centrifuged at 12,000 g for 30 min. The supernatant was chromatographed on a 5.3 cm × 18.5 cm bed of DE-52 (Whatman Inc., Clifton, NJ) that had been equilibrated in DE-52 column buffer. The flow rate was 50 ml/h, and 4-ml fractions were collected. Bound protein was eluted from the column with a linear gradient from 0 to 0.3 M KCl in DE-52 column buffer. The total gradient volume was 2,000 ml. Meta-vinculin-containing fractions were pooled and dialyzed vs. hydroxylapatite column buffer (HAP buffer, 10 mM phosphate buffer, 0.15 M NaCl, 0.02% NaN<sub>3</sub>, pH 7.3). After centrifugation, the supernatant was applied to a 2.1 cm × 7.9 cm bed of HAP resin (Bio-Rad Laboratories, Cambridge, MA) that had been equilibrated in HAP column buffer. This column was run at a flow rate of 30 ml/h, and 3-ml fractions were collected. Addition of 1 mM EGTA and 1 mM EDTA (200 μl each) to each tube in the fraction collector before collection of fractions greatly minimized the proteolysis that otherwise occurred at this step. After washing the column to remove unbound protein, bound protein was eluted from the resin with a linear gradient from 0.01 to 0.3 M sodium phosphate buffer in HAP column buffer (total gradient volume was 300 ml). Fractions containing meta-vinculin were pooled, dialyzed vs. HPLC column buffer (identical to DE-52 column buffer) plus 10% ultrapure sucrose, and then filtered through a 0.2-μm Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, MI). The material was fractionated by HPLC (LKB Instruments, Inc., Gaithersburg, MD) at room temperature on a mono Q anion exchanger (Pharmacia Fine Chemicals, Piscataway, NJ). Bound proteins were eluted from the mono Q resin with a gradient from 0 to 0.3 M KCl in DE-52 column buffer (500 ml of each buffer). The flow rate was 1 ml/min and 1-ml fractions were collected. Purified meta-vinculin was stored at 4°C in the presence of PIC I and PIC II.

### Characterization of Anti-Vinculin

Antibodies to chicken gizzard smooth muscle vinculin were purified from rabbit sera by affinity chromatography and characterized as described previously (26).

### Quantitation of Meta-Vinculin and Vinculin Extracted from Smooth Muscle under Different Ionic Conditions

5 g, wet wt, of frozen smooth muscle from gizzards of adult chickens were homogenized at 4°C in a Waring blender with 35 ml of either 2 mM Tris, 1 mM EGTA, pH 9 (low salt buffer) + 10 μl/10 ml PIC I + 10 μl/10 ml PIC II, or 35 ml of 0.6 M Tris, 1 mM EGTA, 1 mM EDTA, pH 7.4 (high salt buffer) containing PIC I and PIC II. The homogenates were stirred for 30 min at 4°C and then centrifuged at 10,000 rpm in an SS-34 rotor for 30 min. A quantitative immunoprecipitation was set up as follows: 0.5 ml of each supernatant was immunoprecipitated with either 30, 60, 80, or 120 μg of affinity purified anti-vinculin (saturating amounts of antibody). The reactions were incubated for 2 h at 4°C. Protein A-Sepharose (Pharmacia Fine Chemicals) was added and the reaction was incubated further for 1 h at 4°C with rocking. The immune complexes were washed sequentially in the following buffers: 50 mM Tris, 5 mM EDTA + 150 mM NaCl, pH 7.5 (TEN) + 0.5% NP-40 + 5 mg/ml BSA; TEN + 0.5% NP-40 + 2.5 M KCl; TEN + 0.5% NP-40; TEN. Immune complexes were pelleted after each wash by a 3-min centrifugation in a microfuge (Beckman Instruments, Inc., Berkeley, CA). Antigen-antibody complexes were eluted from Protein A-Sepharose by the addition of 2× Laemmli sample buffer, boiled for 2 min, and then centrifuged in a microfuge (Beckman Instruments, Inc.) for 3 min. The supernatants were electrophoresed on a 5% SDS polyacrylamide gel (21). The gel was stained with Coomassie Blue, destained, and then pho-

tographed. The negative of the gel was scanned on a densitometer (Pharmacia Fine Chemicals). The amount of vinculin and meta-vinculin immunoprecipitated was determined from a standard curve derived from known amounts of purified vinculin electrophoresed and scanned in the same manner as above.

### Determination of Physical Parameters of Purified Meta-Vinculin

The Stoke's radius of meta-vinculin was determined from its elution volume on gel filtration by the method of Ackers (1). The sedimentation coefficient of meta-vinculin was determined by sedimentation velocity analysis on 5–20% linear sucrose gradients as described by Martin and Ames (22). Two-dimensional electrophoresis was carried out by the method of O'Farrell (24).

### Detergent Binding Assays

Meta-vinculin (a partially purified preparation) was analyzed for its ability to bind detergent by the Triton X-114 phase separation assay as described by Bordier (5).

### Two-dimensional Peptide Mapping

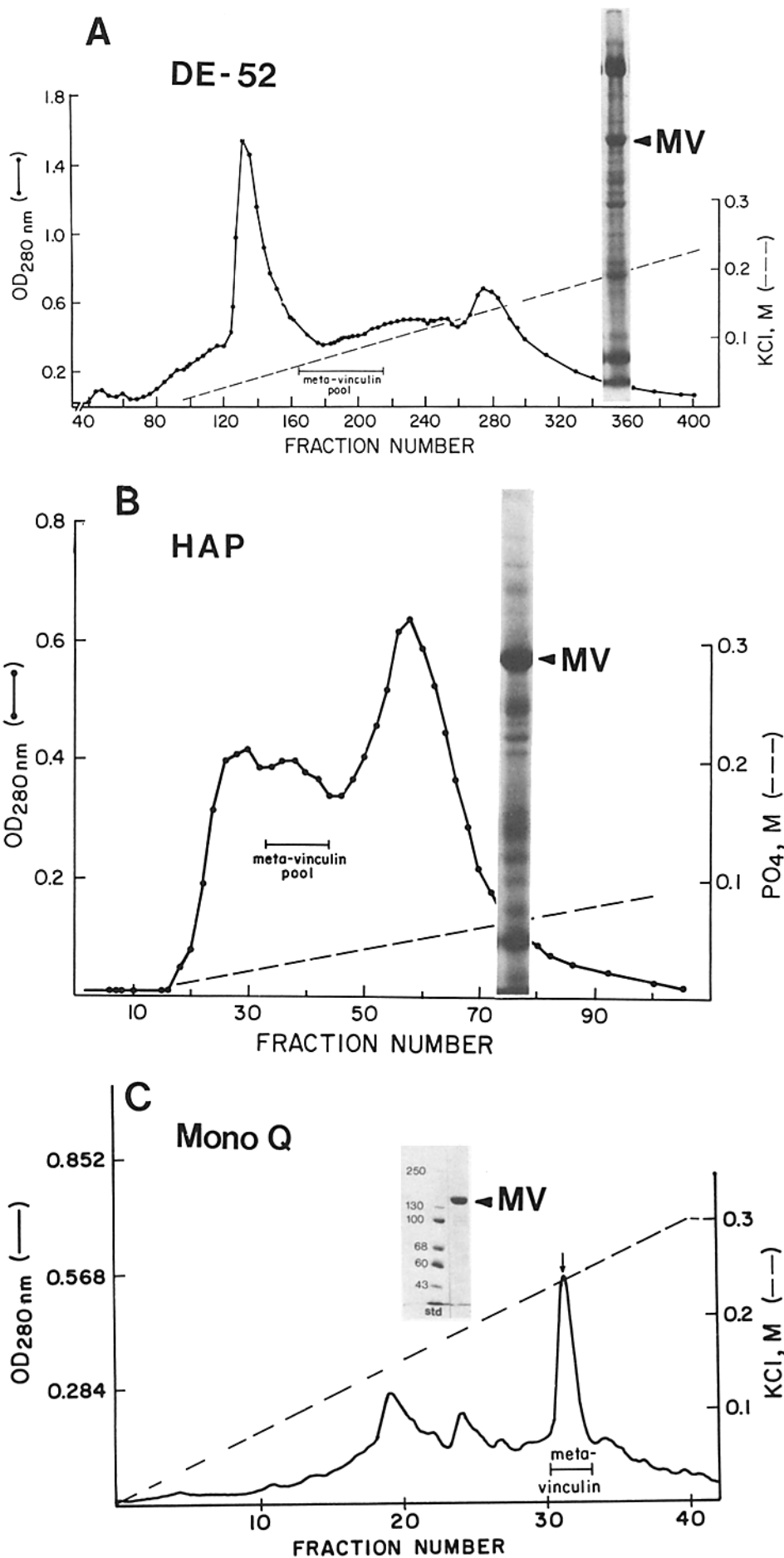
Vinculin, meta-vinculin, and the low-salt-soluble 152-kD protein were immunoprecipitated with affinity purified anti-vinculin. Vinculin and the 152-kD protein were immunoprecipitated from the low ionic strength extract of gizzard smooth muscle, whereas meta-vinculin was immunoprecipitated from the high salt extract of gizzard smooth muscle. All three immunoprecipitates were iodinated in solution by the method of Greenwood et al. (20), and then separated by electrophoresis on a 5% SDS polyacrylamide gel. <sup>125</sup>I-labeled meta-vinculin, vinculin, and 152-kD protein were cut out of the gel and prepared for peptide mapping as described by Elder et al. (11). <sup>125</sup>I-labeled proteins were enzymatically digested with 50 μg/ml chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) for 12 h at 37°C. Peptides were separated electrophoretically in the first dimension in Buffer I (acetic acid/formic acid/H<sub>2</sub>O, 15:5:80), followed by ascending chromatography in the second dimension in Buffer II (butanol/pyridine/acetic acid/H<sub>2</sub>O, 32.5:25.0:5.0:20:0). At least two replicates for each protein were done on the same day using the <sup>125</sup>I-labeled peptides from a single iodination. At least five iodinations were performed and the results were reproducible, although the intensity of some spots varied substantially.

### In Vivo Pulse-Chase Studies

Cells from gizzards of 12-d-old chick embryos were cultured by the method of Campbell et al. (10). Monolayers were pulsed for 1 h with 600 μCi [<sup>35</sup>S]methionine per dish (specific activity = 1,200 Ci/mM; Amersham Corp., Arlington Heights, IL) in methionine-free RPMI medium (Gibco, Grand Island, NY). All cultures were kept at 37°C in a 5% CO<sub>2</sub> humidified incubator. After the pulse, the labeling medium was removed, monolayers were rinsed with complete RPMI medium (Gibco Laboratories) plus 0.2 mM cold methionine, and then chased for various lengths of time in the same medium. At various times during a 5-h chase, monolayers were scraped off with a rubber policeman and homogenized in 1 ml 0.6 M Tris, 1% Triton, PIC I, and PIC II pH 7.4, with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The homogenate was spun at 100,000 g for 60 min and the supernatant immunoprecipitated with anti-vinculin, followed by fixed *Staphylococcus aureus* as described above. The immune complexes

Table 1. Differential Solubility of Meta-Vinculin and Vinculin in High Salt and Low Salt Buffers

Extraction buffer	Amount of protein extracted/5 g smooth muscle	
	Vinculin	Meta-vinculin
	μg	μg
0.6 M Tris, 1 mM EDTA, 1 mM EGTA, pH 7.4	805.0	161.0
2 mM Tris, 1 mM EDTA, pH 9.0	609.0	17.5



**Figure 1.** Purification of meta-vinculin. (A) DE-52 chromatography. Proteins precipitated with 17–30%  $(\text{NH}_4)_2\text{SO}_4$  were dialyzed vs. DE-52 column buffer and chromatographed on DE-52 anion exchange resin. Proteins were eluted from the resin with a 2,000-ml linear gradient of 0–0.3 M KCl in DE-52 column buffer. The inset is a Coomassie Blue-stained, 7.5% SDS polyacrylamide gel of proteins present in 60  $\mu\text{g}$  of the meta-vinculin pool, fractions 165–215 on the column profile. (B) HAP chromatography. Meta-vinculin-enriched fractions 165–215 from the DE-52 column were pooled, dialyzed, and chromatographed on HAP. Proteins were eluted from the HAP resin with a 300-ml linear gradient of 10 mm–0.3 M sodium phosphate, pH 7.3. The inset is a Coomassie Blue-stained, 7.5% SDS polyacrylamide gel of proteins present in 60  $\mu\text{g}$  of the meta-vinculin pool, fractions 33–44 on the column profile. (C) HPLC on mono Q resin. Meta-vinculin-enriched fractions 33–44 from the HAP column were pooled, dialyzed, and then chromatographed using HPLC with a mono Q anion exchanger. Proteins were eluted from the resin with a gradient of 0–0.3 M KCl. Meta-vinculin elutes from this column (fraction 32) at 240 mM KCl. The inset is a 7.5% SDS polyacrylamide gel of 30  $\mu\text{g}$  of protein from fraction 32.

were eluted from the bacteria as described above and electrophoresed on a 5% SDS polyacrylamide gel. The gel was stained, destained, dried, and exposed to preflashed x-ray film.

### Detection of Meta-Vinculin Binding Proteins

Purified meta-vinculin was iodinated by the Bolton and Hunter method (4). [<sup>125</sup>I]Meta-vinculin was used to probe Western blots (33) of purified cytoskeletal proteins and gizzard extracts. Extracts were prepared by homogenizing adult gizzard smooth muscle in either 0.6 M Tris, 1 mM EDTA, 1 mM EGTA, PIC I, and PIC II (pH 7.4), or 0.6 M Tris, 0.5% SDS, 0.1% deoxycholate, 1% NP-40, PIC I, and PIC II (pH 7.4). The extracts were centrifuged at 100,000 g for 60 min. Supernatants were electrophoresed on a 7% SDS polyacrylamide gel. Proteins in the gel were electrophoretically transferred to nitrocellulose paper and then probed with [<sup>125</sup>I]meta-vinculin as described by Baines and Bennett (2).

### In Vivo Phosphorylation Studies

Cell cultures prepared from embryonic gizzards were biosynthetically labeled with [<sup>32</sup>P]orthophosphate (2 mCi/dish, Amersham Corp.) for 4 h at 37°C in phosphate-free DME. Then the cultures were rinsed, scraped off the dish with a rubber policeman, and homogenized with 5 ml 0.6 M Tris, 1 mM EDTA, 1 mM EGTA, PIC I, and PIC II, pH 7.4, using a small Dounce homogenizer (Kontes Glass Co.). The extract was centrifuged at 100,000 g for 60 min. The supernatant was immunoprecipitated with affinity purified anti-vinculin for 2 h on ice. Immune complexes were precipitated with Protein A-Sepharose (Pharmacia Fine Chemicals) and then washed as described above. Antigen-antibody complexes were eluted from the Protein A-Sepharose by boiling for 2 min in twofold concentrated (2×) Laemmli sample buffer. Sepharose beads were pelleted and the supernatants were electrophoresed on a 5% SDS polyacrylamide gel. The gel was stained in Coomassie Blue, destained, and then exposed to x-ray film.

## Results

### Quantitation of Meta-Vinculin under Different Extraction Conditions

In a previous study (30) we showed qualitatively that low salt buffers did not extract meta-vinculin efficiently. In this study, the results of quantitative immunoprecipitation studies (Table I) confirm that there is a substantial difference between the solubility of meta-vinculin and vinculin. The high salt buffer (0.6 M Tris, 1 mM EGTA, pH 7.4) extracts ninefold more meta-vinculin than the low salt buffer (2 mM Tris, 1 mM EDTA, pH 9.0). In contrast, vinculin is extracted only 1.3-fold more efficiently in high salt buffer than low salt

**Table II. Amount of Protein at Various Stages of Meta-Vinculin Purification**

	Protein*
	mg
Starting material: 200 g gizzards, wet wt, 0.6 M Tris, 1 mM EGTA, 1 mM EDTA, pH 7.4 extract (high salt extract)†	19,240
12,000 g supernate of high salt extract after dialysis vs. low salt buffer	7,392
17–30% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitated proteins	2,400
DE-52 column sample	2,300
HAP column sample	76.4
HPLC column sample (mono Q anion exchanger)	15.8
Purified meta-vinculin	3.0

\* Protein values were obtained by a modified Lowry assay (28).

† There is 7.0 mg meta-vinculin in this high salt extract as determined by quantitative immunoprecipitation as described in the text.

buffer. Consequently, meta-vinculin was purified from the high salt extract.

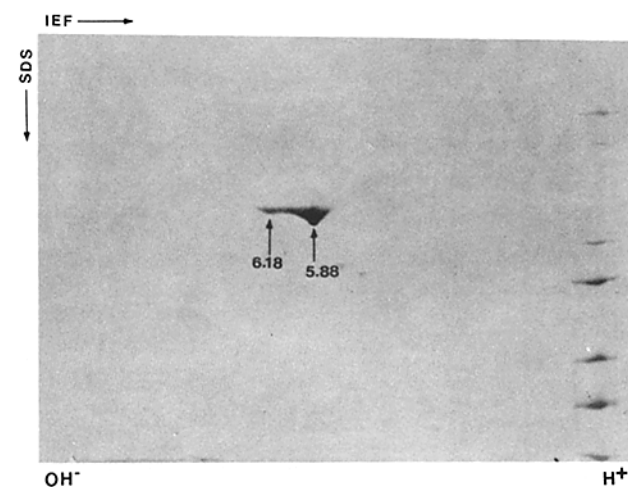
### Purification of Meta-Vinculin from Gizzard Smooth Muscle

Meta-vinculin was extracted from chicken gizzard smooth muscle by 0.6 M Tris. The solubilized material was applied to a column of DE-52 that was eluted with 0–0.3 M KCl. Meta-vinculin elutes from this column between 0.055 and 0.095 M KCl (Fig. 1 A). A representative fraction of the material that was pooled and applied to the next column is shown in Fig. 1 A (inset). The preparation was purified further by HAP chromatography (Fig. 1 B). Meta-vinculin elutes from HAP between 0.025 and 0.035 M sodium phosphate. HAP chromatography separates the major contaminant, filamin (Fig. 1 B, fractions 50–75), from meta-vinculin (Fig. 1 B, fractions 33–48). A gel lane representative of the pooled meta-vinculin containing fractions is shown in Fig. 1 B (inset). This material was purified further by HPLC chromatography using a mono Q anion exchanger. Meta-vinculin elutes from this resin with 0.24 M KCl and is at least 98% pure (Fig. 1 C, fraction 32, and inset). The minor bands that can be observed in an SDS gel (Fig. 1 C, inset) of purified meta-vinculin are proteolytic fragments of meta-vinculin because they cross-react with affinity purified anti-vinculin in a Western blot (data not shown). About 3 mg of highly purified meta-vinculin are obtained from 200 g of gizzard smooth muscle (Table II). This represents a recovery of 43% of the total meta-vinculin present in the high salt extract of gizzard smooth muscle.

### Physical Parameters of Purified Meta-Vinculin

The purity of isolated meta-vinculin is evident from two-dimensional gel analysis (Fig. 2). Meta-vinculin has at least two isoforms. The major isoform has a pI of 5.88, which is more acidic than any of the vinculin isoforms reported (12, 17) or observed in our experiments. The minor meta-vinculin isoform has a pI of 6.18.

The Stokes radius of meta-vinculin was determined by gel



**Figure 2.** Two-dimensional electrophoresis of purified meta-vinculin. Purified meta-vinculin was isoelectrofocused in the first dimension by the method of O'Farrell (24) using 80% pH 5–7 ampholines and 20% pH 3–10 ampholines. The second dimension was electrophoresis on a 7% SDS polyacrylamide gel.

Table III. Determination of Stokes Radius by Gel Filtration

	Elution volume ( $V_e$ )	$K_{av}$	Stoke's radius
	ml		Å
Blue Dextran	33.11	—	—
Bromphenol Blue	96.30	—	—
Thyroglobulin	52.69	0.345	85.0
Catalase	63.64	0.533	52.2
Aldolase	64.50	0.548	48.1
Meta-vinculin	64.10	0.541	50.9
Vinculin	66.45	0.582	45.41

filtration on a calibrated Sepharose 6B column (Table III). Meta-vinculin eluted from the column as a single symmetrical peak with a Stokes radius of 50.9 Å (Table III). In comparison, vinculin had a Stokes radius of 45.41 Å under the same column conditions (Table III). Sedimentation velocity analysis on 5–20% linear sucrose gradients with standard proteins indicated that meta-vinculin had a sedimentation coefficient of 6.35S (Fig. 3). These data show that meta-vinculin is a monomeric protein with a calculated molecular mass of 145,000 D. Table IV summarizes the physical properties of purified meta-vinculin.

#### Meta-Vinculin Does Not Bind Triton X-114

Partially purified meta-vinculin was assayed for its ability to bind Triton X-114 detergent micelles by the method described by Bordier (5). The Triton X-114 phase separation assay has been used to demonstrate the presence of hydrophobic domains in a protein (5). Both vinculin (Fig. 4 B) and meta-vinculin (Fig. 4 A), as well as contaminating proteins present in the partially purified preparations, partitioned into the aqueous phase. In contrast, the integral membrane protein,  $Ca^{2+}$ -ATPase from sarcoplasmic reticulum, did bind Triton X-114 and was found entirely in the detergent phase (Fig. 4 C).

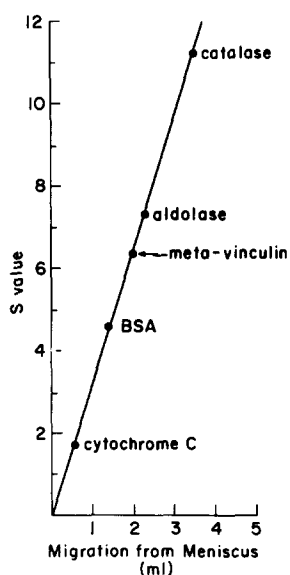


Figure 3. Determination of sedimentation coefficient of meta-vinculin with standards of known S value. The sedimentation coefficient of meta-vinculin was determined from the plot made from standards of known S value: cytochrome C (1.7S), BSA (4.6S), aldolase (7.3S), and catalase (11.2S). The sedimentation coefficient of meta-vinculin is 6.35S.

Table IV. Summary of Physical Properties of Meta-Vinculin

Stokes radius, $R_s^*$	50.9 Å
Diffusion coefficient, $D^\dagger$	$4.15 \times 10^{-7}$ cm <sup>2</sup> /s
Sedimentation coefficient, $s_{20,w}^\ddagger$	6.35S
$M_r$ SDS electrophoresis	150,000 D
$M_r$ calculated from $R_s$ and $s_{20,w}$	145,000 D
Frictional ratio, $f/f_0^\parallel$	1.46

\* From gel filtration on Sepharose 6B in 0.15 M NaCl, 10 mM  $PO_4$ , pH 7.3.  
 † Calculated from diffusion coefficient,  $D = RT/6\pi R_s N \eta$ , where  $N$  is Avogadro's number and  $\eta$  is the viscosity of  $H_2O$  at 20°C.

‡ From sedimentation velocity analysis on linear 5–20% sucrose gradients.

‖ Calculated from frictional ratio,  $f/f_0 = R_s \left( \frac{4\pi N}{3vM_r} \right)^{1/3}$ ;  $v$  was assumed = 0.74.

#### Meta-Vinculin Is Phosphorylated In Vivo

Immunoprecipitation of extracts of <sup>32</sup>P-labeled cells from embryonic gizzards reveal that meta-vinculin is phosphorylated in vivo (Fig. 5 B, lane 1'). From densitometric scans of the Coomassie Blue-stained gels and the corresponding autoradiographs, we calculate that meta-vinculin is phosphorylated in vivo to a specific activity at least eight times greater than that of vinculin (Fig. 5 A). A high molecular mass (>300 kD), <sup>32</sup>P-labeled band can also be observed when <sup>32</sup>P-labeled extracts are immunoprecipitated with anti-vinculin (Fig. 5 B, lane 1'). The identity of this band is unknown and its appearance on gels is variable. Two-dimensional peptide maps show that meta-vinculin has at least one heavily labeled phosphopeptide that is not present in vinculin (data not shown).

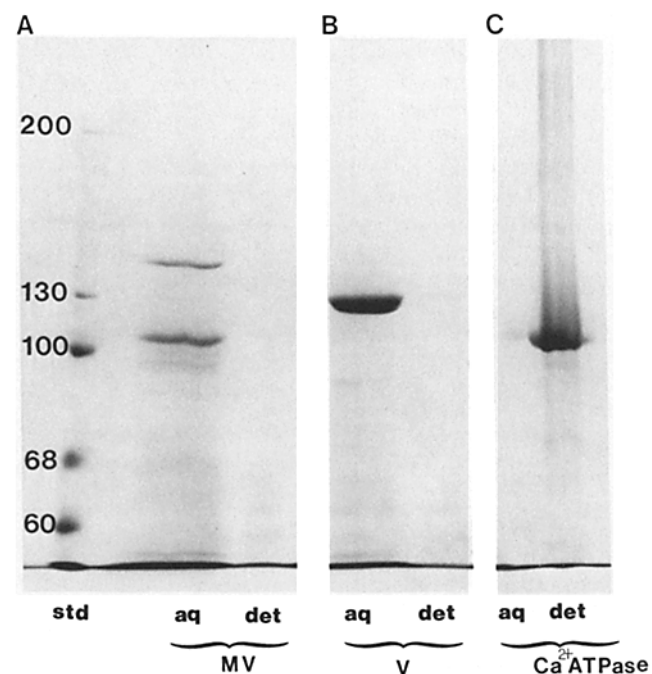
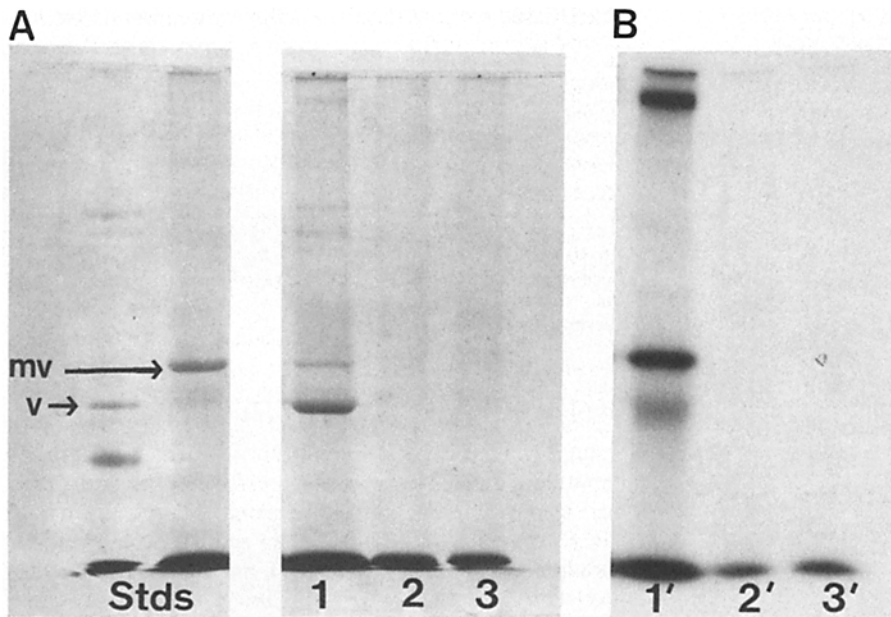


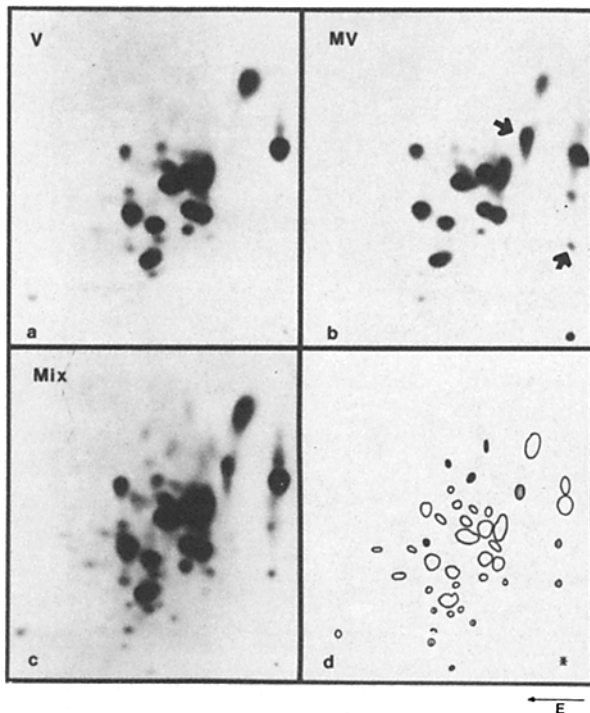
Figure 4. Meta-vinculin does not bind Triton X-114. Proteins were incubated with Triton X-114 at 0°C. The mixtures were then incubated at 30°C, the cloud point of Triton X-114. At 30°C, the detergent separates into an aqueous phase and a detergent-rich phase. The two phases are separated by centrifugation. Meta-vinculin (A) and vinculin (B) are found exclusively in the aqueous (aq) phase. In contrast, the integral membrane protein,  $Ca^{2+}$ -ATPase, partitions into the detergent (det) phase (C).



**Figure 5.** Meta-vinculin is a phosphoprotein. Cell cultures of embryonic chick gizzard were labeled biosynthetically with [ $^{32}$ P]orthophosphate. Vinculin and meta-vinculin were immunoprecipitated from a 0.6 M Tris, pH 7.4 extract of the labeled cells. *A* is a Coomassie Blue-stained gel. Lane 1, proteins immunoprecipitated with anti-vinculin (talin, 190-kD breakdown of talin, vinculin, and meta-vinculin). Lanes 2 and 3 are the controls (lane 2, normal rabbit IgG substituted for anti-vinculin, lane 3, extract plus Protein A). *B* is an autoradiogram of *A*. Lane 1' shows that both vinculin and meta-vinculin are phosphoproteins.

**Two-dimensional Chymotryptic Peptide Maps of Iodinated Vinculin and Meta-Vinculin Are Closely Homologous**

Two-dimensional chymotryptic peptide maps of vinculin and meta-vinculin (Fig. 6) demonstrate a close homology between these two proteins. The two molecules share 95% of



**Figure 6.** Two-dimensional, chymotryptic peptide maps of iodinated vinculin and meta-vinculin. Chymotryptic peptides of vinculin (*a*), meta-vinculin (*b*), and a mix of equal counts of vinculin and meta-vinculin peptides (*c*). (*Open forms*), shared peptides; (*hatched forms*), meta-vinculin (MV) unique peptides; (*solid forms*), vinculin-unique peptides. Arrows mark the major peptides referred to in the text.

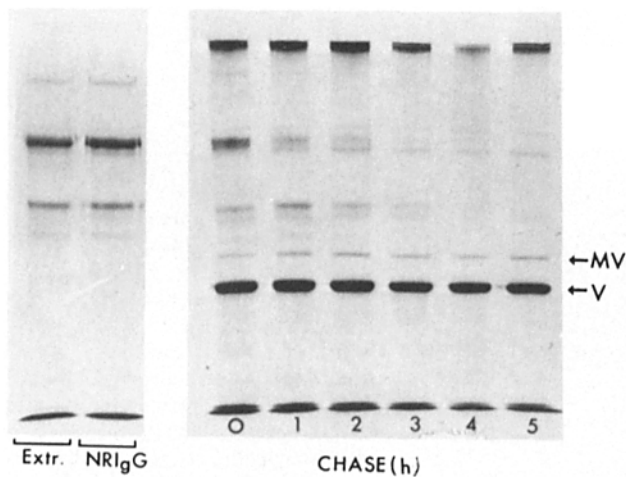
the heavily labeled iodinated chymotryptic peptides. In addition, both vinculin (Fig. 6 *A*) and meta-vinculin (Fig. 6 *B*) have unique peptides. Meta-vinculin has two heavily labeled unique peptides; these unique peptides can be seen in the mix (Fig. 6 *C*) and are reproducible. There is one heavily labeled chymotryptic peptide that is unique to vinculin; this peptide can also be visualized in the mix (Fig. 6 *C*). Similar conclusions as above are obtained if vinculin and meta-vinculin are digested with proteases having different cleavage specificities from chymotrypsin (trypsin and *S. aureus* V8 protease; data not shown).

**In Vivo Pulse-Chase Experiments Show That Vinculin and Meta-Vinculin Do Not Have a Precursor-Product Relationship**

To determine whether vinculin and meta-vinculin are distinct proteins differing in primary amino acid sequence, or whether there is a precursor-product relationship between vinculin and meta-vinculin, in vivo pulse-chase experiments were carried out. The results of this experiment (Fig. 7) indicate that during a 5-h chase, vinculin is not processed to meta-vinculin, nor is meta-vinculin proteolyzed to vinculin. The same results are obtained if vinculin and meta-vinculin are immunoprecipitated at various times, starting at 5 min, during a 1-h chase or after a 24-h chase (data not shown). Thus, unless processing occurs in the 5 min required to lyse and extract the cells, these results suggest that vinculin and meta-vinculin are independent proteins.

**Two-dimensional Chymotryptic Peptide Maps of Iodinated Meta-Vinculin and 152-kD Protein Are Highly Homologous but Not Identical**

Although we found that meta-vinculin is preferentially extracted from gizzards with high salt buffers (30), Feramisco et al. (15) reported that a similar vinculin-like protein can be isolated from a low ionic strength extract of gizzard smooth muscle. Are meta-vinculin and the 152-kD protein the same protein? The two-dimensional chymotryptic fingerprints of the two molecules demonstrate extensive homology between



**Figure 7.** Analysis of precursor-product relationship between vinculin and meta-vinculin by pulse-chase experiments in [<sup>35</sup>S]methionine labeled cells from embryonic chicken gizzard. Vinculin (*v*) and meta-vinculin (*mv*) were immunoprecipitated at various times during a 5-h chase. The other bands that can be visualized are proteins in the extract (*Extr*) that bind nonspecifically to the *S. aureus* bacteria in the absence of antibody. These nonspecific bands are also seen if normal rabbit IgG (*NRIgG*) is used instead of anti-vinculin in the immunoprecipitation.

the two proteins (Fig. 8). Although the presence of at least one major peptide difference suggests that the two proteins are not identical, the peptide maps do not show whether the difference is at the level of amino acid sequence.

#### Detection of Meta-Vinculin Binding Proteins

A modification of the Western blot technique (33), using [<sup>125</sup>I]meta-vinculin as probe, was used to detect meta-vinculin binding proteins. Meta-vinculin only bound to the cytoskeletal protein, talin, when tested on several purified cytoskeletal proteins (Fig. 9 B, lanes 3 and 4). Talin, a 215-kD protein from smooth muscle (6, 7), binds to vinculin *in vitro* (9). The binding of [<sup>125</sup>I]meta-vinculin to talin can be displaced with 100-fold excess of unlabeled meta-vinculin (Fig. 9 C).

Several other meta-vinculin binding proteins in various extracts of chicken gizzard smooth muscle were also identified in a similar manner (Fig. 9, D–F). [<sup>125</sup>I]Meta-vinculin bound specifically to proteins of molecular mass 215, 190, 180, 150, 95, 70, 68, and 45 kD (Fig. 9 E). The 215-kD protein is most likely talin. The identity of the other bands is not known. The binding of meta-vinculin to these gizzard smooth muscle proteins is also displaced by 100-fold excess of unlabeled meta-vinculin (Fig. 9 F).

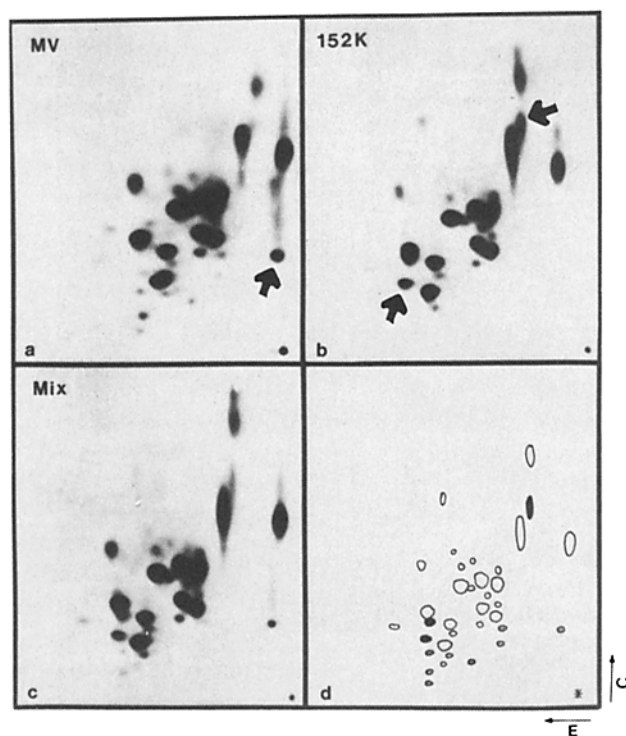
#### Discussion

Development of a protocol for isolation of meta-vinculin in high yield and purity has allowed further characterization of the molecule and better understanding of its possible function in cells. Our earlier results (30) showed that nonionic detergent, in addition to high ionic strength, was required for complete solubilization of meta-vinculin from unfixed smooth muscle cryostat sections. This led us to state that meta-vinculin had the solubility properties of an integral membrane protein. These earlier studies were performed on

unfixed tissue sections that were adherent to glass slides. Apparently adhesion of the tissue to glass slides affects the solubility of meta-vinculin because in the present study meta-vinculin could be extracted efficiently by homogenization of gizzard in high salt buffers in the absence of detergent. Meta-vinculin did not bind to Triton X-114, indicating that the isolated protein does not have a large, accessible hydrophobic domain. Also, purified meta-vinculin does not require detergent to remain soluble. Thus, meta-vinculin may associate peripherally with the plasma membrane of smooth muscle cells.

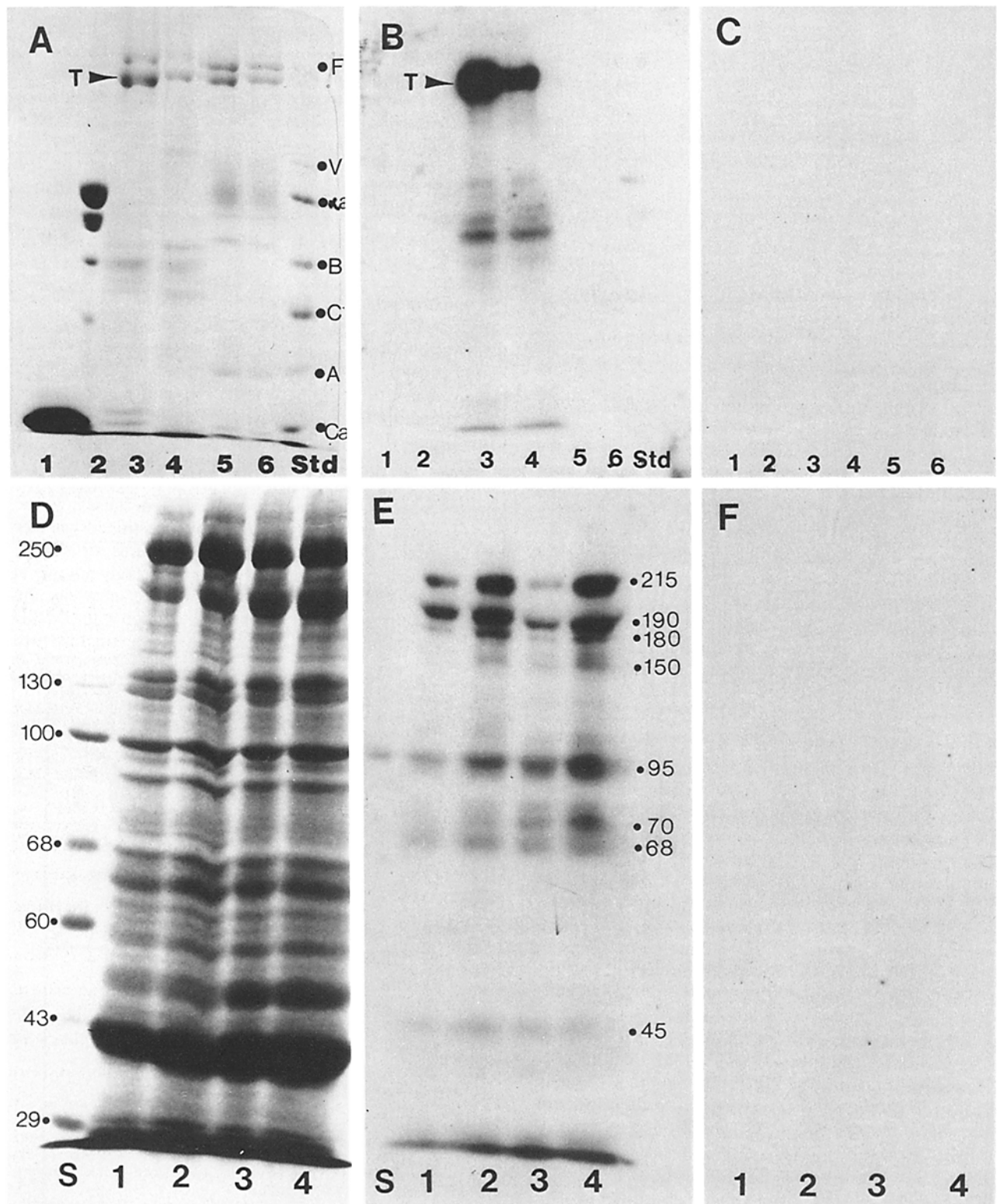
The quantitative analysis of the solubility of meta-vinculin and vinculin in buffers of low versus high ionic strength confirm our earlier qualitative finding that the two proteins have distinct solubilities. The fact that meta-vinculin is more highly phosphorylated than vinculin *in vivo* and that it has a more acidic pI could explain the requirement for a high ionic strength buffer (greater charge shielding) for efficient extraction.

Because the 152-kD vinculin-like protein studied by Feramisco et al. (15) was isolated from low ionic strength buffer, whereas meta-vinculin was discovered (30) because it was so poorly soluble in this same buffer, we questioned whether there were two 150-kD vinculin related proteins, or alternatively, a single species that was simply sparingly soluble at low ionic strength. Therefore, we compared meta-vinculin immunoprecipitated from a high salt extract with 152-kD protein immunoprecipitated from a low salt extract by two-



**Figure 8.** Two-dimensional chymotryptic peptide maps of iodinated meta-vinculin and 152-kD protein. Two-dimensional maps of meta-vinculin (*a*) and (*b*) 152-kD chymotryptic peptides. *c* is a mixture of equal counts of both meta-vinculin and 152-kD peptides. In *a* and *b*, 120,000 cpm were spotted at origin (*asterisk*); in *c*, 240,000 cpm were run. *d* is a sketch of *c*. (*Open forms*), shared peptides; (*hatched forms*), meta-vinculin (*MV*) unique peptides; (*solid forms*), 152-kD unique peptides. Arrows indicate the major peptide differences referred to in the text.





**Figure 9.** Meta-vinculin binding proteins. (A) A Coomassie Blue-stained gel of tropomyosin (lane 1),  $\alpha$ -actinin (lane 2), partially purified talin (arrowhead, lanes 3 and 4), and red blood cell ghost proteins (lanes 5 and 6). (B) A western blot of an identical gel probed with [ $^{125}$ I]meta-vinculin. [ $^{125}$ I]Meta-vinculin binds specifically to talin (B, lanes 3 and 4) and a protein of 70 kD. (C) Binding of [ $^{125}$ I]meta-vinculin can be displaced with 100-fold excess of unlabeled meta-vinculin. (D) A Coomassie Blue-stained gel of proteins extracted from chicken gizzard in 0.6 M Tris, 1 mM EGTA, 1 mM EDTA, pH 7.4 (lanes 1 and 2) or 0.6 M Tris, 0.5% SDS, 0.1% deoxycholate, and 1% NP-40, pH 7.4 (lanes 3 and 4). (E) A Western blot of an identical gel as in D probed with [ $^{125}$ I]meta-vinculin. [ $^{125}$ I]Meta-vinculin binds to proteins of 215 (talin), 190, 180, 150, 95, 70, 68, and 45 kD (panel B, lane 1) in both kinds of extracts. (F) The binding can be displaced with 100-fold excess of unlabeled meta-vinculin.



dimensional maps of iodinated peptides. The chymotryptic maps of 152-kD protein and meta-vinculin show that these proteins are highly related, but suggest that they may not be identical. The unique peptides could represent either sequence differences or posttranslational modifications. The results of the peptide maps imply that either the 152-kD protein is not extracted by high salt or that it comprises a very small amount of the 150-kD vinculin-like protein in the high salt extract. Otherwise, the 152-kD unique peptides would also be detectable in the meta-vinculin peptide map, which is not the case. Another possibility, which we cannot fully exclude, is that the peptide differences represent the presence of small amounts of different contaminants in the immunoprecipitates. However, these contaminants would have to co-migrate with vinculin or meta-vinculin as the maps were made from protein bands that were cut out of gels. Molecular cloning and sequence studies of the vinculin-related proteins will ultimately define their diversity.

Two-dimensional maps of iodinated vinculin and meta-vinculin show that the two proteins are highly related, but pulse-chase experiments demonstrate that they are not related by a precursor-product event. Taken together, these two experiments suggest that vinculin and meta-vinculin are distinct proteins arising from separate mRNAs. Vinculin and meta-vinculin could be encoded by two different genes or they could arise from a single gene whose transcript is spliced differentially. These results confirm the earlier conclusion of Feramisco et al. (15).

In this study we found that meta-vinculin is a monomeric, slightly asymmetric protein with a calculated molecular mass of 145,000 D. By rotary shadowing, vinculin appears to have an 8-nm globular head connected to a slender 20-nm-long tail (23); the shape of meta-vinculin has not been visualized by this technique. Because meta-vinculin and vinculin have a mass difference of 15,000–20,000 D, it will be informative to see how this extra mass is distributed on the meta-vinculin molecule.

In preliminary experiments to assess the function of meta-vinculin in smooth muscle, we have looked for meta-vinculin binding proteins. [<sup>125</sup>I]Meta-vinculin binds in a saturable manner to eight polypeptides in smooth muscle extracts. Of these, the 215-, 190-, 180-, and 45-kD polypeptides have also been detected in [<sup>125</sup>I]vinculin blots (9, 25, 34). The significance of the other four polypeptides is under investigation. Meta-vinculin does not appear to bind to F-actin as determined by co-sedimentation experiments (data not shown). Recent reports suggest that vinculin also does not bind detectably to actin (13, 35).

In summary, vinculin and meta-vinculin are highly homologous proteins. However, differences in their peptide maps, molecular mass, solubility, degree of phosphorylation, and the restricted expression of meta-vinculin in smooth muscle argue for a specialized role for meta-vinculin in the dense plaques of smooth muscle cells.

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