Co-existence of Vinculin and a Vinculin-like Protein of Higher Molecular Weight in Smooth Muscle*

James R. Feramisco‡, John E. Smart§, Keith Burridge¶, David M. Helfman‖, and G. Paul Thomas

From the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Recently, a protein component of adhesion plaques with a molecular weight of 130,000 (named vinculin) has been purified from smooth muscle and non-muscle cells. As detected by immunological methods, the only vinculin-related polypeptides in fibroblasts are proteins of Mr = 130,000. However, we show here that smooth muscle contains, in addition to vinculin, an apparently distinct protein with a Mr = 152,000 that shares both structural and immunological features with vinculin. Amino acid analysis, peptide mapping, and antibody cross-reaction studies were used to elucidate these similarities. Mr = 152,000 protein seems to be restricted to muscle (mainly or exclusively to smooth muscle). The possibility that vinculin is derived from proteolytic processing of the Mr = 152,000 protein or that the proteins are related by some other type of post-translational modification appears unlikely (although this cannot be completely ruled out) since both proteins are made in a rabbit reticulocyte cell-free translation system when mRNA derived from smooth muscle is used as the template. Both proteins are capable of lowering the viscosity of F-actin solutions, although the activity of the Mr = 152,000 protein is stimulated by Ca²⁺ while the activity of smooth muscle vinculin is not.

The molecular organization of the actin filament system in muscle and non-muscle cells has been the object of intense study over the past 10 years. This has led to the discovery of several actin microfilament accessory proteins. A protein of 130 kd in both smooth and non-muscle tissues, named vinculin (1-4), has been shown to be localized at the termini of actin bundles (actin-membrane junctions) (1, 3) and to be colinear with actin bundles close to the dorsal surface of cultured cells, where the extracellular protein fibronectin and the actin bundles are seen to coincide (3). In addition, it has been reported that vinculin, located by immunological techniques, is also found in the dense adhesion plaque regions of smooth muscle (4), another area where actin bundles terminate at the membrane. This intracellular location has led to the suggestion that vinculin is involved in the organization of actin bundles at their membrane attachment sites or more directly in the attachment of actin bundles to the membrane. Vinculin does not seem to be present in isolated plasma membrane preparations from HeLa cells (6) and such cells contain actin filaments, rather than bundles, in association with their membranes (6). This observation might suggest that this protein is involved in the organization of the highly ordered bundles of actin microfilaments that are found in well spread cells, whereas in rounded cells (such as HeLa cells in suspension) it assumes a lesser role in the arrangement of individual microfilaments which associate with the membrane. In vitro biochemical studies have shown that vinculin isolated from smooth muscle causes a decrease in the viscosity of F-actin solutions (7-9). In the case of vinculin isolated from non-muscle HeLa cells, this activity is stimulated by Ca²⁺ (8).

In the course of fractionating smooth muscle, according to a scheme developed in this laboratory designed for the purification of a-actinin, filamin, and vinculin (2), an additional protein of Mr = 152,000 was detected. The 152-kd protein eluted in an initial chromatographic step (DEAE-cellulose) between those fractions enriched for filamin and those enriched for a-actinin (2). It occurred to us that the 152-kd protein might also constitute a structural element of muscle tissue. We therefore employed immunofluorescence localization using antisera raised against the 152-kd protein to survey its occurrence in different tissues. Non-muscle cells were also examined because, in many cases, counterparts to the structural components of muscle cells are also found. We describe here our findings on the immunological and structural characteristics of the 152-kd protein.

We show that the 152-kd protein, found only in muscle (smooth and non-muscle) tissues, shares extensive homology with the 130-kd protein vinculin, which is found in both muscle and non-muscle tissues. The two proteins share antigenic features as revealed by reciprocal cross-reactivity and direct structural analyses indicated a high degree of relatedness. The results of cell-free translation of mRNA, amino acid analysis, and two-dimensional gel electrophoresis make it likely that the 152-kd protein and vinculin are independent gene products, rather than constituting a precursor-product couple or some other posttranslationally modified pair.

At a biochemical level, the 152-kd protein shares with vinculin the ability to decrease the viscosity of F-actin. Unlike the muscle form of vinculin, however, Ca²⁺ causes a marked stimulation of this activity of the 152-kd protein. This finding, coupled with their different tissue distributions, may imply that they have distinct functional roles.

**EXPERIMENTAL PROCEDURES**

*Protein Purification*—The initial steps in the purification of the 152-kd protein from chicken smooth muscle (gizzard) are identical with those used in the preparation of vinculin described previously.
by our laboratory (2). Briefly, 50 g of frozen chicken gizzard was homogenized in and washed with 0.5 mM phenylmethylsulfonyl fluoride and extracted at 37 °C with a low ionic strength buffer (pH = 9). The extract was treated with 10 mM Mg²⁺ to induce the precipitation of most of the actin and desmin and then fractionated by ammonium sulfate-induced precipitation. The precipitate obtained from the 38% ammonium sulfate saturation curve was dialyzed against Buffer B (20 mM Tris/acetate (pH 7.6), 20 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol) and applied to a DEAE-cellulose column. Whereas vinculin is obtained in a homogeneous form after chromatography on DEAE-cellulose, the 152-kd protein requires two additional steps. These are described under "Results."

**Antibody Production**—Antibodies against the 152-kd protein were made by the subcutaneous immunization of rabbits with the protein which was further purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10) of the hydroxypatite column fractions. For the initial injection, 200 µg of the 152-kd protein was emulsified in 1 ml of Freund's complete adjuvant; four subsequent boosts were done with 200 µg of protein emulsified in Freund's incomplete adjuvant over a 2-month period. The immune serum (diluted 1:100) generated from this protocol stained chick embryo fibroblasts by immunofluorescence, as well as established lines of fibroblasts (Swiss 3T3, gerbil fibroma), with a punctate pattern corresponding to the adhesion plaques of the cells and thus was similar to immune sera made against vinculin (3; see "Results").

**Immunological Procedures**—Immunofluorescence localization of vinculin and the 152-kd protein and photomicrography of stained cells have been described previously (3). Cells for immunofluorescence (Fig. 2) were derived from embryonic chicken gizzard by digestion with trypsin as described below for skin fibroblasts. Samples for the SDS-gel analysis or immunoprecipitation of vinculin and the 152-kd protein from tissues were prepared as follows. Day 12 chick embryos were dissected to yield breast tissue (skeletal muscle), gizzard (smooth muscle), heart (cardiac muscle), and skin (fibroblasts). The skin was treated with 0.05% trypsin, 1 mM EDTA, in phosphate-buffered saline for 15 min at 37 °C to free the fibroblasts. The cells were collected by centrifugation and plated onto plastic dishes and subcultured in 10% fetal calf serum in Dulbecco's modification of Eagle's medium. Each tissue sample was washed in phosphate-buffered saline and homogenized in 10 volumes of hot SDS-gel electrophoresis sample buffer (11) and boiled for 5 min.

For immunoprecipitation, the boiled samples were diluted to a final SDS concentration of 0.1% by the addition of 1% deoxycholate and 1% Triton X-100 in phosphate-buffered saline. After preabsorption of the samples with 10 mg of IgG-Sorb (readily sedimentable form of Staphylococcus aureus Protein A) (The Enzyme Center), 2 µl of preimmune anti-vinculin or anti-152-kd protein serum was added. The solution was incubated for 6 h at 4 °C and then mixed with 10 µg of IgG-Sorb. The pellets containing the antibody-antigen complexes were washed 3 times with 1% deoxycholate, 1% Triton X-100, 0.1% SDS in phosphate-buffered saline and disrupted by boiling in SDS-gel sample buffer (5 min). The samples were analyzed by SDS-polyacrylamide gel electrophoresis using either a gradient of acrylamide (12) or the formulation of Blatter et al. (13) with the buffer system of Laemmli (11). The gels were processed for and visualized by fluorography (14). For the identification of antigens in the tissues, the elution from hydroxyapatite exchange chromatography of the 152-kd protein by gel filtration through Sephacryl S-300 was used. The major antibody was found to be anti-vinculin or anti-152-kd protein followed by 111I-labeled goat anti-rabbit IgG.

**RNA Preparation**—Total cell RNA was prepared from chicken embryonic (day 12) smooth muscle (stomach and gizzard) and from HeLa cells by a modified guanidine thiocyanate procedure. Tissues or pelleted suspension cells were homogenized in guanidine thiocyanate (16) and diluted with equal volumes each of redistilled phenol, 0.1 M sodium acetate (pH 5.0), and chloroform/isoamyl alcohol (28.1/1, v/v) and adjusted at 60 °C. After changing to fresh phases, the upper (aqueous) phase was reextracted with half-volumes each of phenol and chloroform/isoamyl alcohol at 60 °C, and twice with chloroform/isoamyl alcohol at room temperature. After two alcohol precipitations, the RNA was chromatographed twice on oligo(dT)-cellulose (Collaborative Research). This step separates protein- and DNA-free high molecular weight RNA in high yield and in a biologically active state as evidenced by its ability to directly synthesize of high molecular weight polypeptides (see Fig. 5). Two-dimensional gel electrophoresis was performed as described by O'Farrell (27).

**Cell-free Translation**—Polyadenylated RNA, added at 10 µg/µl, was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (30) in reaction mixtures for 40 min at 30 °C, after which the reaction was treated with pancreatic ribonuclease (20 µg/ml) and EDTA (20 mM) for 20 min at 37 °C, then diluted with an equal volume of 2 × SDS sample buffer in preparation for gel electrophoresis or immunoprecipitation.

**Peptide Mapping**—Gel slices containing labeled polypeptides were located by autoradiography or fluorography and processed for the generation of tryptic peptide maps as described in detail elsewhere (18). Briefly, performic acid oxidations and tryptic digestions were performed within microcr gel slices and peptides eluted in 0.1 x NH₄HCO₃. Lyophilized peptides were dissolved in formic acid, diluted with H₂O to 20% formic acid, and chromatographed on a Spherisorb 10 ODS (C₁₈) reverse-phase column using a gradient of 0-62.5% ethanol in 4.5% formic acid. Fractions of 0.7 ml were mixed with 4.2 ml of Aquasol (New England Nuclear) and counted by liquid scintillation spectrometry. Alternatively, "native" peptide maps were generated by incubation of the purified proteins with S. aureus V-8 protease (Miles Laboratories). Aliquots of the reaction mixture were mixed with an equal volume of 2 × SDS sample buffer, boiled, and analyzed by gel electrophoresis.

**Viscometric Analysis**—Low shear falling ball viscometry (19) was used to analyze the interactions of vinculin and the 152-kd protein with 10 mM Mg²⁺ or 10 mM Ca²⁺ at 37 °C. The results of these experiments were as follows:

**RESULTS**

**Purification of the 152-kd Protein**—Chromatography through DEAE-cellulose of the smooth muscle proteins extracted by the low ionic strength/37 °C procedure (2) separates the 152-kd protein from vinculin (Mr = 130,000) but not completely from filamin (Mr = 280,000), a-actinin (Mr = 100,000), and actin (Mr = 45,000). The bulk of the contaminating filamin and a-actinin is removed from the 152-kd protein by gel filtration through Sephacryl S-300 while some, but not all, of the actin is removed (Fig. 1A). Ion exchange chromatography of the 152-kd protein purified by microprep was used to identify the majority of the contaminating actin (Fig. 1C), giving rise to a highly purified preparation of the 152-kd protein. More recent preparations of the 152-kd protein have been made using a somewhat shallower salt gradient in the elution from hydroxyapatite (i.e. 0-200 mM potassium phosphate versus 0-400 mM as shown here) and contain less actin as a contaminant. This procedure yielded 1-2 mg of protein from 50 g of starting material. Upon storage, the 152-kd protein undergoes cleavage to generate a 100-kd product in addition to other smaller polypeptides. This 100-kd fragment is clearly derived from the 152-kd protein as seen by tryptic peptide analysis (Fig. 4).

**Immunofluorescence Staining of Fibroblasts with Antibodies against the 152-kd Protein**—The purified 152-kd protein was subjected to preparative SDS-polyacrylamide gel electrophoresis to ensure that a homogeneous polypeptide was in hand (10). This material was used to elicit antibodies in two rabbits, both of which gave rise to similar antisera. While preimmune rabbit serum showed no discrete staining of chick embryo gizzard cells (not shown), the immune sera showed the punctate patterns of staining that coincided with the termini of the actin stress fibers (Fig. 2, A and B). This pattern is strikingly similar to the pattern obtained when antibodies against vinculin are used (1, 3) (Fig. 2, C and D). To test whether or not the staining pattern revealed by the anti-152-kd serum was due to antibodies that recognize the 152-kd...
130-Kd and 152-Kd Vinculin-like Proteins

Fig. 1. Purification of the 152-kd protein from smooth muscle. In each panel, samples of the indicated column fractions were analyzed by SDS-polyacrylamide gel electrophoresis (11, 12) and stained with Coomassie blue. The positions of molecular mass markers (from top to bottom: 200 kd, 116 kd, 94 kd, 68 kd, and 45 kd) are indicated by the lines on the right of each panel. A. DEAE-chromatography. Material precipitating at 28–38% ammonium sulfate was applied to a DEAE-cellulose column and eluted with a linear gradient of 0 (fraction 4)–370 mM (fraction 100) sodium chloride in Buffer B. B, Sephacryl S-300 chromatography. Fractions 50–56 from the DEAE-cellulose step were pooled and dialyzed against 70% w/v sucrose in Buffer B. The concentrated protein was redialyzed against Buffer B and applied to a Sephacryl S-300 (Pharmacia Fine Chemicals) column equilibrated in Buffer B (1.2 × 100 cm). One column volume (bed volume) was collected in 100 fractions and the indicated fractions were analyzed by SDS-gel electrophoresis. C, hydroxyapatite chromatography. Fractions 50–54 from the S-300 column were pooled and applied to a hydroxyapatite (Bio-Rad HTP) column (1.2 × 15 cm) equilibrated in Buffer B. Elution of the proteins was with a linear potassium phosphate (pH 7.5) gradient from 0 (fraction 3)–400 mM (fraction 60). Fractions 18–24 were pooled and concentrated against sucrose as above. This procedure yielded 1–2 mg of protein from 50 g of starting material.

Fig. 2. Immunofluorescence staining of cultured gizzard cells with anti-vinculin and anti-152-kd sera. Phase (A, C, E, G) and fluorescence (B, D, F, H) micrographs of chicken embryo gizzard cells stained with rabbit anti-152-kd serum (A, B), rabbit antivinculin (C, D), anti-152-kd serum after preabsorption with purified vinculin (1 mg/ml, 30 min) (E, F), and anti-vinculin after preabsorption with purified 152-kd protein (1 mg/ml, 30 min) (G, H). In all cases, the immune sera were diluted 1:100 (final). The coverslips were treated secondarily with fluorescein-labeled goat and anti-rabbit IgG (1:50) (Cappel Laboratories) to visualize the primary antibody (antigen) distribution. Exposure times for all the fluorescence micrographs were restricted to the same time of approximately 10 s.
staining patterns given by their respective antisera (not shown), which was as expected. Surprisingly, though, it was also found that preabsorption of anti-152-kd serum with vinculin (Fig. 2, E and F) and preabsorption of anti-vinculin serum with the 152-kd protein (Fig. 2, G and H) abolished the staining patterns of both sera.

**Tissue Distribution of the 152-Kd Protein**—To determine the occurrence of the 152-kd protein in various tissue types, embryonic chicken parts (day 12) were examined by immunoprecipitation or by the antibody-mediated SDS-gel staining technique (15) using both anti-vinculin and anti-152-kd sera. Smooth muscle and fibroblasts contained relatively more vinculin than cardiac and skeletal muscle. These immunological methods further showed that in relative terms smooth muscle contained the most 152-kd protein, cardiac and skeletal contained trace amounts, and embryonic fibroblasts contained no detectable 152-kd protein. We estimate that there is about 5 times more vinculin than 152-kd protein in smooth muscle, as judged from these results and from the relative yields of the two proteins during purification. Identical results were obtained using either anti-vinculin or anti-152-kd protein sera: both antisera recognized the same two proteins in these tissues. A sample experiment is shown in Fig. 3. It is not yet clear if the trace amounts of the 152-kd protein found in cardiac and skeletal muscle originate from these tissues or from contaminating smooth muscle in the tissue preparations (e.g., vascular smooth muscle). It should also be noted that the relative amount of vinculin present in skeletal muscle was found to be lower in the adult chicken as opposed to the day 12 embryo, a result which may indicate the importance of the

![Fig. 3. Immunological identification of the 152-kd protein and vinculin in different tissues. Day 12 chick embryos were dissected to yield breast tissue (skeletal muscle), gizzard (smooth muscle), heart (cardiac muscle), and skin (fibroblasts). The skin was treated with 0.05% trypsin, 1 mM EDTA, in phosphate-buffered saline for 15 min at 37°C, to free the fibroblasts. The cells were then collected by centrifugation and plated onto plastic dishes and subcultured in 10% fetal calf serum in Dulbecco's modification of Eagle's medium. Each tissue sample was washed in phosphate-buffered saline and homogenized in 10 volumes of hot SDS-gel electrophoresis buffer (11) and boiled for 5 min. Two-ml aliquots of each tissue were run on a 10% SDS-polyacrylamide gel and either visualized by Coomassie blue stain (A) or by autoradiography using the antibody-mediated staining technique (15) with rabbit anti-vinculin (B) or rabbit anti-152-kd protein (C) followed by 125I-labeled goat anti-rabbit IgG. Slot I shows molecular mass markers (same as in Fig. 1), slots 2-7 show skeletal muscle, cardiac muscle, smooth muscle, fibroblasts, vinculin, and 152-kd protein, respectively, in the three panels.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Vinculin</th>
<th>152-kd protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn</td>
<td>9.1</td>
<td>9.7</td>
</tr>
<tr>
<td>Thr</td>
<td>5.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Ser</td>
<td>5.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Gln</td>
<td>9.8</td>
<td>12.2</td>
</tr>
<tr>
<td>Pro</td>
<td>7.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Gly</td>
<td>6.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Ala</td>
<td>11.4</td>
<td>12.4</td>
</tr>
<tr>
<td>Val</td>
<td>7.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Met</td>
<td>4.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Ile</td>
<td>4.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Leu</td>
<td>8.7</td>
<td>9.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Phe</td>
<td>2.7</td>
<td>1.7</td>
</tr>
<tr>
<td>His</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Lys</td>
<td>7.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Arg</td>
<td>5.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Cys</td>
<td>2.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Corrected for 10% loss during hydrolysis.
* Determined as cysteic acid.
* Not determined.

![Fig. 4. Peptide mapping of "native" vinculin and the 152-kd protein. A SDS-polyacrylamide gel analysis of the digestion products of vinculin and the 152-kd protein obtained by the action of S. aureus V-8 protease is shown. Ten µg of purified vinculin (tracks a-e) or 10 µg of purified 152-kd protein (tracks f-j) dissolved in Buffer B was incubated alone (tracks e and j) or with 0.3 µg of S. aureus V-8 protease for 60 min (tracks a and f), 5 min (tracks b and g), 20 min (tracks c, f, and h), or 30 min (tracks d and i). The reactions were quenched by the addition of an equal volume of hot SDS-gel sample buffer, heated at 100°C for 3 min, and loaded onto a 15% SDS-polyacrylamide gel. Molecular weight markers (Bio-Rad) (track k) were, from top to bottom, 200,000, 116,000, 94,000, 68,000, 40,000, 30,000, 21,000, and 14,000.](http://www.jbc.org/)
state of differentiation of this tissue in the expression of the protein.

Amino Acid Compositions of Vinculin and the 152-Kd Protein—Purified vinculin and 152-kd polypeptides were subjected to acid hydrolysis followed by the determination of their amino acid compositions. As can be seen in Table I, the 152-kd polypeptide was found to be largely similar to vinculin, except for higher contents of certain amino acids (Thr, Ser, Glx) and lower contents of Pro, Cys, and Lys. (By isoelectric focusing analysis, the 152-kd protein focuses to a more acidic position than does vinculin (see Fig. 7), and this may well reflect this increased acidic content.)

Peptide Mapping Studies—In light of the extremely similar nature of vinculin and the 152-kd protein revealed by antibody cross-reactivity (Figs. 2 and 3), the two proteins were compared at a peptide level. This was done in a variety of ways. First, the purified “native” proteins were completely digested in 30 min at 37 °C by S. aureus V-8 protease added at 1:30 (w/w) the amount of the purified proteins. The peptides generated by such a reaction were examined by SDS-polyacrylamide gel electrophoresis (Fig. 4). Both vinculin and the 152-kd protein yielded a major fragment of 90–100 kkd, with vinculin giving rise to an additional 27-kd fragment. The 152-kd protein, on the other hand, gave rise to an additional fragment with a Mr = 21,000.

Secondly, purified vinculin and 152-kd protein were iodinated using the chloramine-T method in the presence of SDS (20) and recovered in preparative SDS-polyacrylamide gels. Their tyrosine-containing peptides, recovered after total digestion by trypsin, were analyzed by reverse-phase high pressure liquid chromatography and appeared essentially indistinguishable in terms of mobility and relative recovery (Fig. 5, A and B). Based upon the amino acid composition of vinculin and the relative abundance of tyrosine (0.6 mol %), we would expect to find (at most) 12 peptides that contain tyrosine. The results of the high pressure liquid chromatography analysis suggest that we have recovered ~8 peptides, further suggesting that this technique is not exhibiting the entire polypeptide structure of these proteins. Parenthetically, a map of the 100-kd fragment found in “aged” preparations of the 152-kd protein showed that it indeed is related to the 152-kd protein but is devoid of a major tyrosine-containing peptide eluting between tubes 83 and 86 (Fig. 5C).

Since tyrosine is a relatively infrequent amino acid in these proteins (Table I), an alternative approach was therefore employed wherein the 152-kd protein and vinculin were metabolically labeled by incubating explanted 9–10-day embryonic gizzards in medium containing radioactive amino acids. These were solubilized in SDS and the proteins were recovered by immunoprecipitation using anti-vinculin serum followed by SDS-polyacrylamide gel electrophoresis. Tryptic peptide maps of the proteins labeled with either [35S]methionine or [3H]leucine are shown in Fig. 5, D–G.

The methionine-containing tryptic peptides of the two proteins were very similar with respect to mobility and yield. There was, however, a weakly retained peptide unique to the 152-kd protein eluting in tubes 10–15, and possibly another unique peptide eluting at around tube 85. The [3H]leucine

![Image of peptide maps](https://example.com/peptide-maps.png)
labeled proteins gave the maps shown in panels F and G. Comparison of vinculin and the 152-kd protein showed peptides eluting between tubes 105 and 110 and between 130 and 135 which are unique to the 152-kd protein. Other than these two peptides, the leucine-containing tryptic peptides were virtually identical in terms of yield and mobility. These comparative structural analyses confirmed that the two proteins are indeed similar.

**Cell-free Translation Studies**—To explore the possibility of a precursor-product relationship between the 152-kd protein and vinculin, the products of cell-free translation of mRNA from appropriate tissues were compared. Nuclease-treated reticulocyte lysate (17) was programmed with polyadenylated RNA extracted from secondary cultures of embryonic fibroblasts, embryonic smooth muscle (gizzard and stomach), or from whole embryos and the vinculin-related products recovered by immunoprecipitation with anti-vinculin serum (Fig. 6).

Fibroblast mRNA directed synthesis of vinculin but not 152-kd protein (track 4), whereas smooth muscle mRNA programmed synthesis of both vinculin and the 152-kd protein (track 5). This was in accord with their tissue distributions determined by immunological methods (Fig. 3). When whole embryo RNA was translated, in addition to vinculin, a small amount of 152-kd protein was detected: the decreased ratio of the 152-kd protein to vinculin may reflect the decreased relative amount of smooth muscle in whole embryo compared with purer sources (gizzard and stomach). When translated in the same tube, an equal mixture of smooth muscle RNA and fibroblast RNA programmed the synthesis of both vinculin and the 152-kd protein (track 7). Neither vinculin nor the 152-kd protein was immunoprecipitated from lysate reactions to which no exogenous RNA had been added (track 8). Further examination of the polypeptides synthesized from the smooth muscle mRNA by two-dimensional gel electrophoresis showed that translation products that co-migrate with purified vinculin and the 152-kd protein are made in this system (Fig. 7). Thus, the tissue distribution of vinculin and the 152-kd protein were the same when assayed at a protein level (Fig. 3) or by cell-free translation of mRNA from different sources.

**Effect of Vinculin and the 152-Kd Protein on the Viscosity of F-Actin**—We were stimulated to investigate the biochemical interaction of vinculin and the 152-kd protein with actin.

---

**Fig. 6. Immunoprecipitation of vinculin-related polypeptides from cell-free translation reactions programmed with mRNA from different tissues.** Polyadenylated RNA was prepared from secondary chick embryo fibroblasts, embryonic smooth muscle (gizzard and stomach), or whole day 12 embryos by chromatography or oligo(dT)-cellulose. RNA was added to nuclease-treated reticulocyte lysate (17) at around half-saturating RNA levels. Translation products were labeled with [35S]methionine. Reaction mixtures were diluted with 2 x SDS-gel sample buffer, boiled (3 min), and brought to 0.1% SDS (final concentration) by the addition of 1% deoxycholate and 1% Triton X-100 in phosphate-buffered saline. After preabsorption of the samples with 10 mg of IgG-Sorb (a readily sedimentable form of S. aureus protein A; the Enzyme Center), 2 µl of either preimmune, anti-vinculin, or anti-152-kd serum was added. The solutions were incubated for 6 h at 4°C and then mixed with 10 µg of IgG-Sorb. The pellets containing the antibody-antigen complexes were washed three times with 1% deoxycholate, 1% Triton X-100, and 0.1% SDS in phosphate-buffered saline and disrupted by boiling (5 min) with an equal volume of 2 x SDS-gel sample buffer. Samples were analyzed by SDS-gel electrophoresis in a 7.5–15% gradient gel (tracks 1–9) or in a 12.5% gel (tracks 4–8), both of which were processed for fluorography (14). Track 1 shows endogenous reticulocyte lysate products and tracks 2 and 3 show complete reaction products directed by chicken fibroblast and smooth muscle RNAs, respectively. Tracks 4 to 8 illustrate the cell-free products immunoprecipitated using anti-vinculin serum from reactions programmed with mRNA from fibroblasts (track 4), smooth muscle (track 5), whole embryos (track 6), or an equal mixture of smooth muscle and fibroblast mRNA (track 7). Endogenous lysate products brought down by anti-vinculin serum are shown in track 8. Tracks 4 to 8 represent 10–20-fold longer fluorographic exposures of reactions of comparable size shown in tracks 1 to 3.

**Fig. 7. Two-dimensional gel electrophoresis of cell-free translation products of smooth muscle mRNA.** Nuclease-treated reticulocyte lysate was programmed with polyadenylated RNA from smooth muscle as described under “Experimental Procedures” and Fig. 6. [35S]Methionine was used to label the newly synthesized products. The reactions were quenched by the addition of SDS-gel sample buffer, followed by heating for 3 min, and then freeze-dried. The samples were dissolved in two-dimensional gel sample buffer to which 10 µg each of vinculin and the 152-kd protein had been added and electrophoresed as described by O’Farrell (27). Isoelectric focusing was from left (acid end) to right (basic end) (3.5–10 pH gradient), and SDS-gel electrophoresis was from top to bottom (12.5% acrylamide gel). Molecular weight markers were as described in the legend to Fig. 4. The gel was first stained with Coomassie blue, photographed, and then processed for fluorography. A shows the Coomassie blue-stained gel; B shows the fluorogram. The arrows point to the positions of the 152-kd protein and vinculin. The other spots are derived from reticulocyte proteins.
with 152-kd protein, as expected, also failed to give a discrete staining pattern. Anti-152-kd serum preabsorbed with vinculin and 152-kd proteins.

It was found that the 152-kd protein, like vinculin, was capable of decreasing the viscosity of F-actin solutions, as measured by low shear viscometry. In the case of smooth muscle cells, however, an unequivocal localization of either the 152-kd protein or vinculin is not possible, a result of the cross-reactivity of the two antisera. One group has already reported the intracellular distribution of vinculin in fibroblasts since only this protein is capable of decreasing the viscosity of F-actin solutions, as measured by low shear viscometry. In the case of smooth muscle cells, however, an unequivocal localization of either the 152-kd protein or vinculin is not possible, a result of the cross-reactivity of the two antisera. One group has already reported the intracellular distribution of vinculin in fibroblasts since only this protein is capable of decreasing the viscosity of F-actin solutions, as measured by low shear viscometry.

**DISCUSSION**

The protein vinculin has been shown to be present in adhesion plaques and sometimes along actin bundles near the dorsal surface of fibroblasts (1, 3); the location of the related 152-kd protein remains to be established. Immunofluorescence staining with anti-152-kd serum or anti-vinculin reveals the localization of vinculin in fibroblasts since only this protein is present (Fig. 2). In smooth muscle cells, however, an unequivocal localization of either the 152-kd protein or vinculin is not possible, a result of the cross-reactivity of the two antisera. One group has already reported the intracellular distribution of vinculin in smooth muscle using an anti-vinculin serum (4), but these findings may reflect the disposition of either or both vinculin and 152-kd proteins.

To avoid such ambiguity, we attempted preabsorption of the sera prior to immunofluorescence staining. Anti-152-kd serum was incubated with excess vinculin or the 152-kd protein purified from SDS gels and, following centrifugation, used to stain cultured embryonic gizzard cells by immunofluorescence. In this way, it had been hoped that preabsorption with vinculin would render the anti-152-kd serum specific for the 152-kd protein; this serum, however, generated essentially no discrete staining patterns. Anti-152-kd serum preabsorbed with 152-kd protein, as expected, also failed to give a discrete pattern of staining. The absorption of the anti-vinculin serum with either 152-kd protein or vinculin completely abolished the immunofluorescence staining pattern normally obtained with this serum.

Biochemical evidence that vinculin interacts with actin filaments to reduce the viscosity of such solutions has recently been obtained from analyses of the interaction by low and high shear viscometry, equilibrium binding of vinculin and F-actin, and by electron microscopy (7-9). The mechanism by which vinculin acts to reduce the viscosity of actin solutions is not as yet understood, but it is likely that vinculin binds to the end(s) of F-actin filaments and thereby prevents further elongation. Vinculin purified from HeLa cells requires Ca2+ for this activity, whereas vinculin from smooth muscle causes a similar decrease in the apparent viscosity of actin solutions. As is the case with the non-muscle (HeLa) form of vinculin, this activity of the 152-kd protein is stimulated by Ca2+. In light of these data, we may speculate that the 152-kd protein provides a Ca2+-sensitive function in smooth muscle that is not provided by the smooth muscle form of vinculin. It may prove necessary to have both a Ca2+-sensitive and a Ca2+-insensitive form of vinculin in muscle since the contraction is accompanied by Ca2+ influx: the 152-kd protein may well provide such a Ca2+-sensitive function.

A precise description of the intracellular distribution of the 152-kd protein will be integral to the definition of the functional aspects of this protein and further attempts are currently under way in our laboratory. Ideal reagents would be monoclonal antibodies specific for either vinculin or the 152-kd protein, and such antibodies used in combination with techniques for microinjection of living cultured cells (21) could not only clarify their cellular locations but also indicate in vivo functions for these proteins. Additionally, experiments involving the microinjection of fluorescently labeled structural proteins into living cells (3), which are free from certain problems inherent in immunological localization techniques, are being carried out.

Cell-free translation of mRNA (Fig. 6) revealed a tissue distribution similar to that found by immunological methods (Fig. 3) and would suggest that expression of the two proteins is regulated at the level of RNA rather than by processing of a polypeptide precursor. These studies, however, cannot exclude a precursor-product relationship between the 152-kd protein and vinculin or some other type of post-translational modification of one of the polypeptides since there are known examples where specific proteases or perhaps even other modification activities are synthesized in active form in cell-free translations and which process other reaction products (22, 23). In the case of a possible precursor-product pair, it would be necessary to imagine either a (fibroblast?)-specific protease which cleaves the 152-kd protein to vinculin or a smooth muscle-specific inhibitor of such an activity, both of which are produced in active form in the reticulocyte lysate. Such a situation is rendered unlikely by the finding that both proteins were produced among the translation products of naturally (whole embryo) or artificially (fibroblast plus smooth muscle) mixed RNA populations. Immunoprecipitation of the cell-free translation products directed by mRNA from an epithelial source (HeLa cells) detected synthesis of vinculin alone (not shown) thereby narrowing even further the restricted tissue distribution of the 152-kd expression.

Vinculin and the 152-kd protein were found to be highly related structurally (Figs. 4 and 5) and thus far indistinguishable antigenically (Fig. 3) yet are probably distinct translation products (Fig. 6 and 7) with distinct biochemical properties (Fig. 8). Despite the differences in apparent molecular weights, about 20,000, few tryptic peptides unique to either polypeptide
were detected. Analysis of tyrosine-containing peptides by reverse-phase chromatography revealed identical patterns although the 152-kd protein could be expected to contain around half as many again iodinated tryptic peptides as vinculin based on their amino acid compositions (Table I) if tyrosine residues were evenly distributed throughout the molecule. Examination of the tryptic peptide maps of the 152-kd protein and vinculin metabolically labeled with either \[^3H\]leucine or \[^35S\]methionine also revealed extensive homology, although a few 152-kd-specific peptides were detected. In the absence of further analysis, which ultimately will probably have to proceed to the level of sequencing, we tentatively suggest that these represent the portion of the 152-kd protein that is unique. The question then arises of whether they are the products of separate but related genes or the products of a single gene but derived through differential processing, perhaps in a manner analogous to the production of membrane-bound and secreted forms of immunoglobulin heavy chains (24-26). This analogy may well extend to other cases where multiple related proteins comprising a common core portion, as well as unique portions, perform distinct yet similar roles and are generated from a single gene. In the case of the 152-kd protein and vinculin, at least, DNA cloning experiments in progress in this laboratory should resolve this point.

Acknowledgments—We thank James D. Watson for enthusiastic support of this work and Kurt Drickamer for performing the amino acid analyses.

REFERENCES

Co-existence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle.
J R Feramisco, J E Smart, K Burridge, D M Helfman and G P Thomas