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Microheterogeneity of Avian and Mammalian Vinculin

Distinctive Subcellular Distribution of Different Isovinculins

BENJAMIN GEIGER

Department of Chemical Immunology
The Weizmann Institute of Science
Rehovot 76100, Israel

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Vinculin from chicken gizzard and from pig heart may be separated by two-dimensional gel electrophoresis into several isoelectrophoretic forms. Peptide map analysis and immunochemical comparison of the different isovinculins indicated that all the isoforms are closely interrelated at the molecular level. Moreover, it was shown that avian and mammalian vinculins have similar molecular structures. Some differences were detected between the isovinculin pattern in intact chicken gizzard tissue and that found in cultured cells from the same organ. Various degrees of vinculin microheterogeneity were also detected in a variety of cultured cells, including primary cultures and several cell lines. Labelling of chicken gizzard cells with [^{32}P]orthophosphate resulted in the incorporation of ^{32}P in the minor acidic isoform of vinculin (α-vinculin) exclusively. Extraction of the cultured cells with detergent under conditions that remove the cytoplasmic "soluble" vinculin without significantly affecting focal contact-associated protein, indicated that specific vinculin isoforms may differ in their cellular distribution. The soluble fraction contained almost exclusively the basic form (β-vinculin), while the "organized" protein contained all three major isovinculins but was enriched with the acidic form (α) and the intermediate form (α'). The functional significance of isovinculin diversity and the involvement of phosphorylation events in vinculin interactions are discussed.

1. Introduction

Vinculin is a 130,000 M_r cytoskeletal protein that is specifically associated with the termini of microfilament bundles in focal contacts of cultured cells (Geiger, 1979; Feramisco & Burridge, 1980*b*). *In vivo*, vinculin was detected in association with several intercellular junctions, including *Zonula adherens* of intestinal epithelium, dense plaques of smooth muscle and intercalated discs of cardiac muscle. Immunoferritin labelling of ultrathin frozen sections of the various tissues indicated that vinculin is closely associated with the cytoplasmic faces of the plasma membrane in those sites (Geiger *et al.*, 1980,1981; Tokuyasu *et al.*, 1981). It was therefore postulated that vinculin is involved in the linkage of actin bundles to the inner surfaces of the plasma membrane (Geiger, 1981,1982; Geiger *et al.*, 1980).

Immunofluorescent labelling of different cell types with specific antibodies showed that vinculin may be organized in three major forms within the cells. Most

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of the labelling in well-spread fibroblasts was associated with focal contacts formed between the ventral cell membrane and the substrate, as well as with fibrillary structures along the actin bundles. In addition, significant amounts of vinculin were present in the cytoplasm in a diffuse, easily extractable form (Geiger, 1981,1982).

During attachment and spreading, as well as during locomotion, cultured fibroblasts continuously form new focal contacts under the leading lamellae, while the posterior contacts fade and detach (Abercrombie *et al.*, 1971; Abercrombie & Dunn, 1975; Izzard & Lochner, 1976,1980). It was postulated that, at the molecular level, this process proceeds in several temporal steps. First, small close contacts (direct or indirect) may be formed between putative membrane receptor(s) and the substrate. Once such local contacts are established, the relevant receptors may cluster to form an "adhesive patch". It was proposed that this clustering induces, across the membrane, assembly of vinculin, which then acts as an organizing centre for the formation of actin filament bundles (Geiger, 1981,1982). This hypothetical model was further corroborated by the report that vinculin induces actin bundle formation *in vitro* (Jockush & Isenberg, 1981). Apparently, one of the critical molecular events in the proposed contact-induced transmembrane signalling is the transition of vinculin from the cytoplasmic, diffusely organized state into membrane-bound clusters. The direct demonstration of this process and its detailed characterization at the molecular level may thus be of great importance for the understanding of the mechanism of cell contact formation. To analyse the possible molecular basis for the polymorphic cellular distribution of vinculin, I have studied the molecular heterogeneity of vinculin itself.

I report here on the existence of several isoelectrophoretic forms of vinculin in tissues and cultured cells. All the isoforms of vinculin have an apparently identical molecular weight (130,000) and a closely related tryptic peptide profile. They differ in the degree of phosphorylation and in their differential association with either the membrane and cytoskeleton-bound or the "soluble", cytoplasmic pools of cellular vinculin. The implications of these results on the mechanisms of contact-induced cytoskeletal rearrangements are discussed.

2. Materials and Methods

(a) *Purification of vinculin*

Chicken gizzard vinculin was purified using either the original purification procedure (Geiger, 1979) or the purification procedure of Feramisco & Burridge (1980*a*). Vinculin preparations purified by both techniques showed the same degree of purity and an identical profile of the isoelectrophoretic forms (isovinculins). Vinculin from pig heart was purified by a similar procedure. The major difference in the purification was that the initial extraction of the tissue was carried out at pH 8·0 to 8·2 (instead of 7·6). Both vinculins were >95% pure, as estimated from sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

(b) *Biosynthetic protein labelling in cultured cells and tissue slices*

Cells were cultured on 60 mm tissue culture dishes (Falcon, U.S.A.) at about 60% confluency in DME (Dulbecco's Modified Eagle's) medium containing 10% (v/v) FCS (foetal calf serum). The cells were incubated for 30 min with methionine-low medium (DME medium with only 0·3 μg methionine/ml and 10% dialysed FCS) and then for 3 to 24 h with 80 to 150 μCi of [^{35}S]methionine (~700 Ci/mmol: The Radiochemical Centre, Amersham, U.K.) in 2 ml of

methionine-low medium. The cells used in these experiments were chicken gizzard fibroblasts (prepared as described; Geiger, 1979), human foreskin fibroblasts (a gift from Dr D. Rotman of this Institute), Henle 407 (human intestinal epithelial cells, CC16; from the American Tissue Culture Collection). PtK_2 cells were obtained from Dr W. Franke, DKFZ, Heidelberg, and human epidermal carcinoma cells A-431 were originally obtained from Dr G. Todaro. Neuroblastoma B-104 was provided by Dr U. Z. Littauer from this Institute.

Slices of chicken gizzard were prepared from hatching chicks. The smooth muscle tissue was dissected and fat and connective tissue were removed. Slices of about 1 mm × 0·5 mm × 0·5 mm were prepared under sterile conditions using type 11 scalpel blades, in the presence of methionine-low medium. The tissue (2 or 3 pieces) was then transferred into wells of flat-bottom microtitre plates (Falcon, U.S.A.) and incubated for 5 h with 0·2 ml of methionine-free DME medium containing 20 μCi of [^{35}S]methionine (as above). Biosynthetic labelling with ^{32}P was carried out in phosphate-free DME medium containing 10% dialysed FCS. The cells were incubated for 12 h with 70 μCi of [^{32}P]orthophosphate/ml (PBS11; The Radiochemical Centre, Amersham, U.K.).

(c) *Immunochemical reagents and methods*

Antibodies to chicken gizzard vinculin were prepared as described (Geiger, 1979). Immunofluorescence labelling was carried out either on cells fixed with 3% (w/v) paraformaldehyde and then permeabilized with 0·2% (v/v) Triton X-100 or on cells that were pre-extracted for 2 min with 50 mM-2-(*N*-morpholino)-ethane sulphonic acid (pH 6·0), 5 mM-$MgCl_2$, 3 mM-ethyleneglycol-bis(β-aminoethyl ether)*N*.*N*′-tetraacetic acid (EGTA), 0·5% Triton and then fixed. The immunofluorescent labelling was indirect, using rhodamine-labelled goat anti-rabbit immunoglobulin as the secondary reagent (Geiger & Singer, 1979).

Immunoprecipitation of vinculin from cells and tissue slices was carried out using the *Staphylococcus aureus* method of Kessler (1975) as described (Geiger, 1979). In some experiments, I have used immunobeads (BioRad, U.S.A.) to which goat anti-rabbit immunoglobulin was conjugated, instead of *S. aureus*. These methods had comparable efficiency and the same specificity. It should be pointed out that, whereas the cultured cells were readily solubilized in 0·2 ml of RIPA (50 mM-Tris·HCl, 150 mM-NaCl, 0·1% (w/v) sodium dodecyl sulphate, 1% (w/v) deoxycholate, 1% (v/v) Triton X-100, pH 7·2) buffer, the tissue slices had to be homogenized in this buffer using small glass/Teflon homogenizers.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was usually carried out on 10% (w/v) slab gels using the Laemmli buffer system (Laemmli, 1970). Two-dimensional gel separation was performed according to O'Farrell (1975) using Pharmalyte ampholines (Pharmacia, Sweden) with IEF (isoelectric focusing) gel, and 10% polyacrylamide gel for the second dimension. The cylindrical gel of the first dimension was cut and the pH in the slices determined. Routine p*I* markers were actin, α-actinin and bovine serum albumin. Extracts of [^{35}S]methionine or ^{32}P-labelled cells were routinely mixed with pure chicken gizzard vinculin prior to their application on the first dimension (IEF) gels. The Coomassie blue spots of the different isovinculins were used as internal markers for the precise localization of the labelled proteins. The slab gels were usually stained with Coomassie blue, the locations of the major isovinculins were marked on them and they were then subjected to dimethyl sulphoxide/PPO intensification according to Bonner & Laskey (1974).

Iodinated tryptic peptide maps were prepared essentially as described by Elder *et al.* (1977). The peptide mapping in this study was performed on 20 cm × 20 cm cellulose plates (Merck, W. Germany).

3. Results

(a) *Microheterogeneity of chicken smooth muscle vinculin*

The microheterogeneity of pure chicken gizzard vinculin was analysed by two-dimensional gel electrophoresis. Usually, five isoforms of the protein were resolved, ranging in p*I* from 6·8 to 7·3 (Fig. 1). I have divided them for the sake of

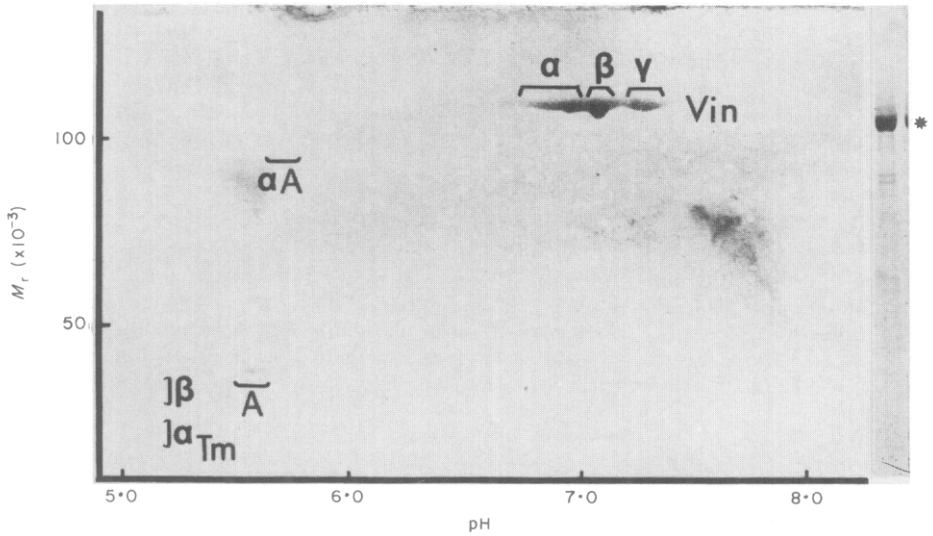

FIG. 1. Two-dimensional gel electrophoresis of pure chicken gizzard vinculin. On the right (marked with an asterisk): a single dimension polyacrylamide gel electrophoretic separation of vinculin. Marked on the gel are the 3 major isovinculins (Vin) α, β and γ, and the locations of α-actinin (αA), actin (A) and the 2 chains of chicken gizzard tropomyosin (Tm) α and β. The gel was stained with Coomassie blue.

convenience into three groups, denoted α, β and γ (the latter being the most basic). Within both the α and β regions, I have usually detected two isovinculin spots, which I refer to as α and α' or β and β', respectively. An identical isovinculin pattern was obtained regardless of the exact purification procedure used or the degree of purity. Nevertheless, in order to ascertain that the multiplicity of vinculin spots was not an artefactual consequence of purification, I have tested the heterogeneity of vinculin that was biosynthetically labelled by chicken gizzard slices. Total RIPA lysates of the [^{35}S]methionine-labelled tissue as well as vinculin immunoprecipitate prepared from this extract contained all three major [^{35}S]methionine-labelled isoforms (Fig. 2) in proportions similar to those found in the purified protein. Quantitative analysis indicated that the approximate relative amounts of the isoforms were: γ, 10 to 30%; β, 50 to 70%; and α, 10 to 20%. These values were obtained by direct measurements of the relative radioactivities in the three zones in the immunoprecipitated, gel-separated vinculin (Fig. 2(b)). In addition to vinculin, I often immunoprecipitated specifically a more acidic component of $\sim$160,000 M_r. This polypeptide had a tryptic peptide map similar to that of vinculin (not shown), and it may be related to the component described by Feramisco *et al.* (1981). It should be pointed out that all the isovinculins exhibited similar antigenic properties and the pattern of immunoprecipitated vinculin was apparently identical to that found in crude extracts of the gizzard tissue (Fig. 2(a)).

(b) *Microheterogeneity of pig heart vinculin*

Purified vinculin from a mammalian source (pig heart) was also found to be polymorphic, though its three major isoforms were significantly more acidic than

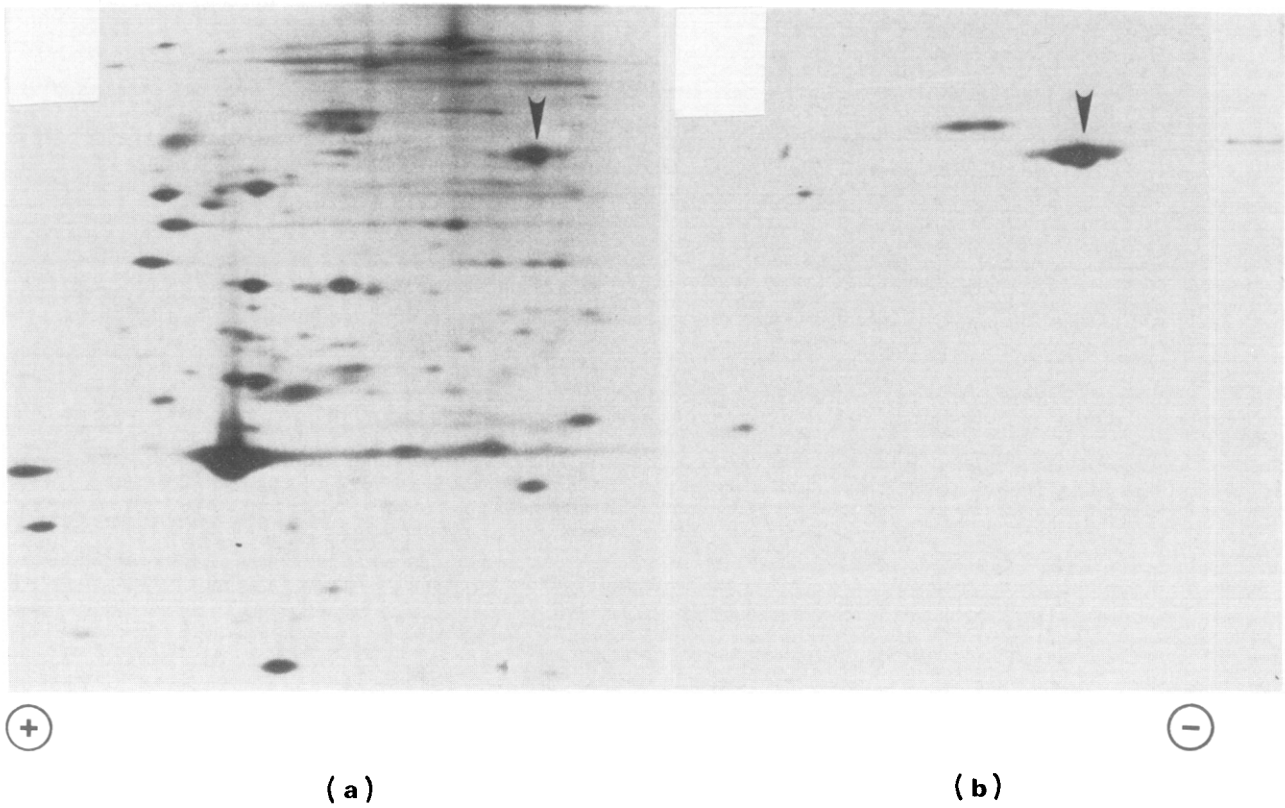

FIG. 2. Autoradiogram of 2-dimensional gel electrophoresis of [^{35}S]methionine-labelled chicken gizzard slices. The labelled tissue was extracted with RIPA buffer and the soluble extract subjected to the isoelectrophoretic separation (a) directly or (b) after immunoprecipitation using vinculin antibodies. The column on the right shows the same immunoprecipitated vinculin, separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (single dimension). Pure chicken gizzard vinculin was added to the sample as an endogenous marker and the location of β vinculin is marked with arrowheads.

those of chicken gizzard (Fig. 3). Thus, the isoelectric points of the pig heart vinculins α, β and γ ranged from p*I* 6·4 to 6·8, respectively. The charge differences between the avian and mammalian isovinculins were further corroborated by co-electrophoresis of chicken gizzard and pig heart vinculin on the same gel as shown in Figure 3(b).

It should be pointed out that, although the chick and the pig isovinculins were similarly designated (α, β and γ) for the sake of convenience, I do not imply that the molecular basis for the variability of isovinculins of the two species is the same.

(c) *Structural relationships between isovinculins: peptide map analysis*

The different isovinculins described above are closely related proteins both antigenically and structurally. The structural analysis was performed by peptide mapping of individual isovinculin spots, which were initially separated by two-dimensional electrophoresis. The gel slices were iodinated, subjected to trypsin and the labelled peptides mapped in two dimensions on cellulose plates. The results (Fig. 4(a) to (c)) indicated that all the isoforms of chicken gizzard vinculin exhibit a closely related peptide pattern, with only few significant variations. I am aware of the fact that the method used here reveals only part of the tryptic peptides (the iodinatable ones). Nevertheless, the high degree of similarity strongly suggests that only limited molecular variation exists between the isoforms tested.

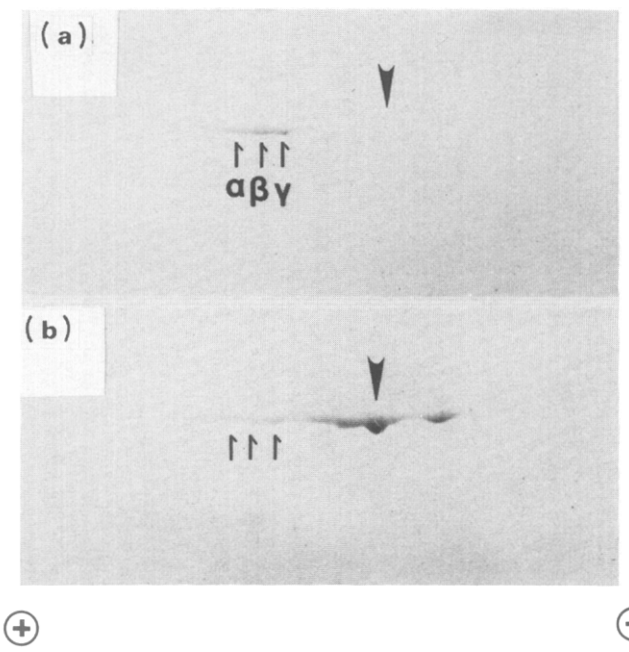

FIG. 3. Two-dimensional gel electrophoresis of pig heart vinculin (a) alone or (b) mixed with chicken gizzard vinculin. The upward-pointing arrows indicate the 3 major pig heart isovinculins α, β and γ. The arrowheads point to the location of chicken gizzard β vinculin.

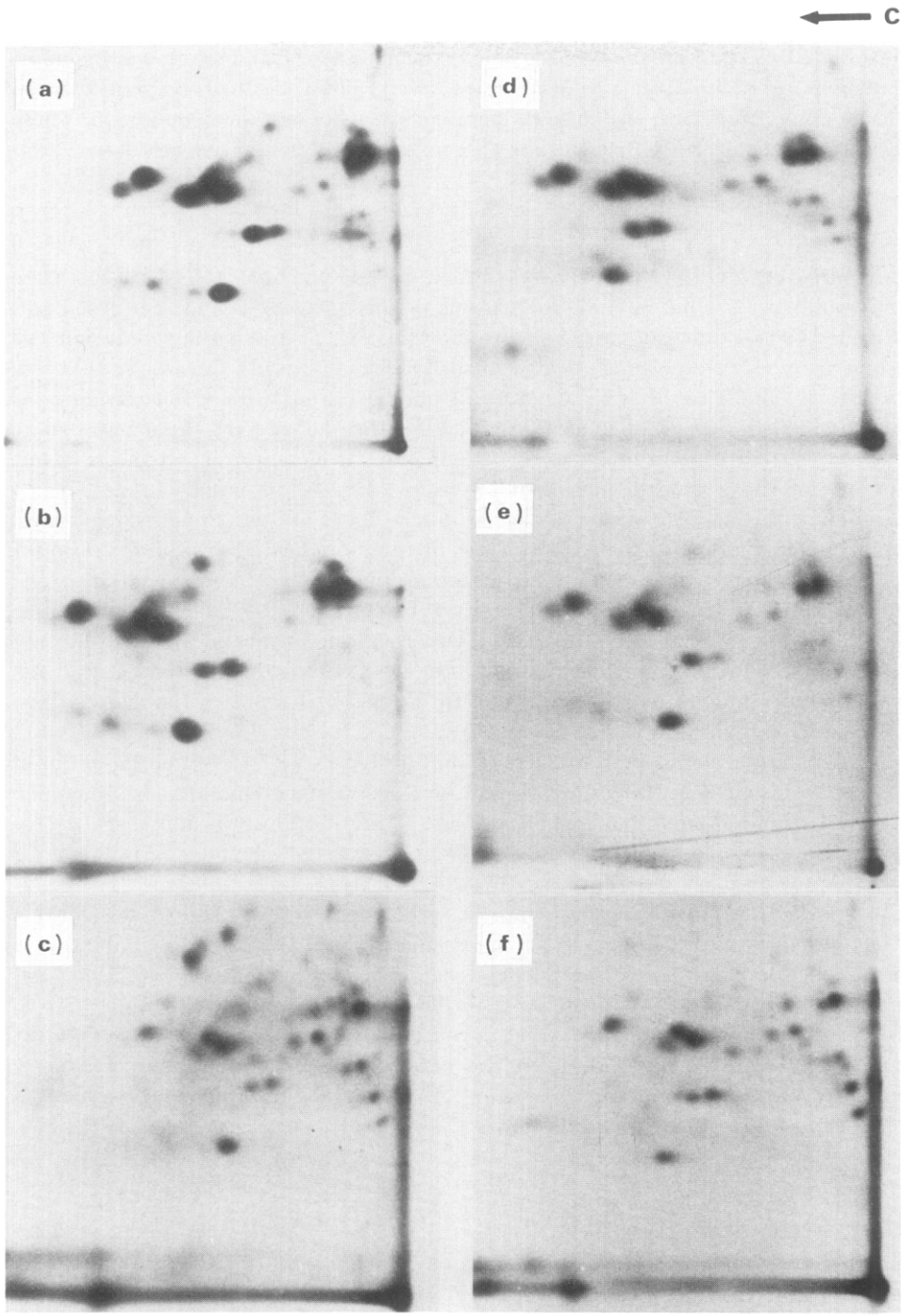

FIG. 4. Iodinated tryptic peptide maps of different isoforms of chicken gizzard ((a) to (c)) and pig heart ((d) to (f)) vinculin. Individual spots from 2-dimensional gel electrophoresis (as in Fig. 3) were excized, iodinated and subjected to complete cleavage by trypsin. (a) Chicken gizzard α vinculin; (b) chicken gizzard β vinculin; (c) chicken gizzard γ vinculin; (d) pig heart α vinculin; (e) pig heart β vinculin; (f) pig heart γ vinculin. The direction of electrophoresis (E) and chromatography (C) are marked.

Similarly, the peptide maps of pig heart isovinculins α, β and γ were very similar, as demonstrated in Figure 4(d) to (f), respectively. Comparison of the peptide maps of vinculins from the two species pointed to a remarkable similarity. These observations suggest that vinculin is an evolutionarily conserved protein.

(d) *Vinculin isoforms in cultured cells*

Cultured chicken gizzard cells synthesize several vinculin isoforms. Cells were incubated with [^{35}S]methionine for various periods (3 to 24 h) and then lysed and subjected to two-dimensional gel electrophoresis (Fig. 5(a)). Similar separation was also performed with biosynthetically labelled chicken gizzard cell vinculin that was specifically precipitated with vinculin antibodies (Fig. 5(b)). Co-electrophoresis with unlabelled, pure chicken gizzard vinculin indicated that the three major spots in these cells correspond to β, α′ and α isovinculins. I have not detected significant amounts of the γ isoform in cultured cells, and thus it is possible that chick γ vinculin is a smooth muscle specific form that is not expressed *in vitro*. Quantitative analysis of the relative concentrations indicated an isoform ratio for β : α′ : α of 70 to 80 : 10 to 20 : 5 to 10, assuming that the methionine content in the various isoforms is the same. The pattern of biosynthetically labelled isovinculins and their apparent relative amounts were not significantly affected by the immunoprecipitation (compare Fig. 5(a) and (b)). It should be pointed out that the length of labelling (3 or 24 h) had no effect on the relative proportions of the different isovinculins.

The labelling of cultured human foreskin fibroblasts with [^{35}S]methionine for 3 to 24 hours resulted in the production of the three major mammalian isovinculins, which are found in pig heart; namely γ, β and α (Fig. 6). This was demonstrated by electrophoretic separation of either crude cell extract or vinculin isolated by immunoprecipitation (Fig. 6(a) and (b), respectively).

In view of the possibility that the observed heterogeneity of vinculin isoforms reflects the cellular heterogeneity in the tissue or in the cell culture, I have tested the isovinculin pattern in several defined cell lines, including human epithelial cell line A-431 and rat neuroblastoma B-104. The results shown in Figure 7 point to cell-type specific variations in the heterogeneity of vinculin. Nevertheless, in most of the cells tested I have detected the major spot (usually the basic form of vinculin), and one or two additional spots. All the mammalian vinculins were more acidic than the avian protein, as shown above for the pure pig protein (Fig. 3).

(e) *Phosphorylation of vinculin* in vivo

Considering the possibility that phosphorylation events play a role in the induction of vinculin organization (see Discussion), I have determined the phosphorylation of this protein *in vivo*. To ensure steady-state labelling, chicken gizzard cells were incubated for 3 to 12 hours with [^{32}P]orthophosphate and then subjected to immunoprecipitation and two-dimensional gel separation. Figure 8 shows the autoradiogram of the ^{32}P-labelled immunoprecipitate (Fig. 8(b)) and the

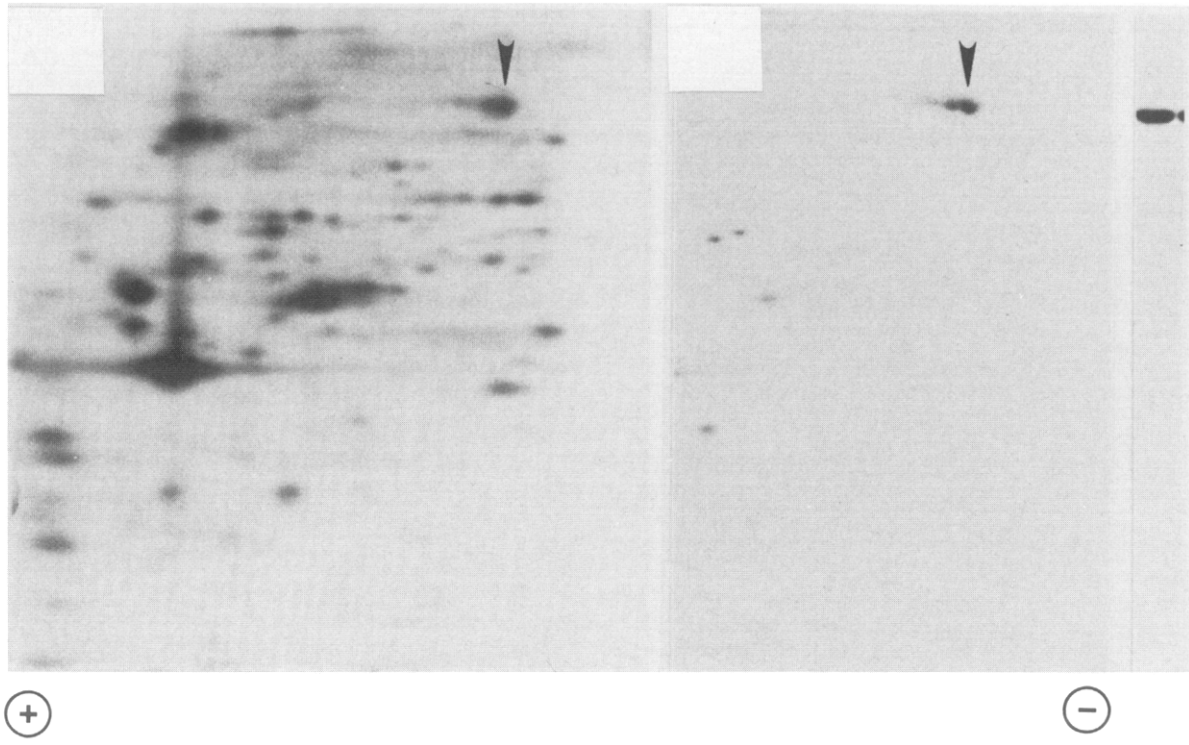

FIG. 5. Autoradiograms of 2-dimensional gel electrophoresis of vinculin from [^{35}S]methionine-labelled cultured chicken gizzard cells (16 h). (a) Total cellular extract and (b) the immunoprecipitate with vinculin antibodies show 3 vinculin isoforms, corresponding to the β (marked with arrowheads), the α' and the α forms of the pure chicken vinculin. The column at the right shows a single-dimension electrophoresis of the chicken gizzard cells vinculin immunoprecipitate.

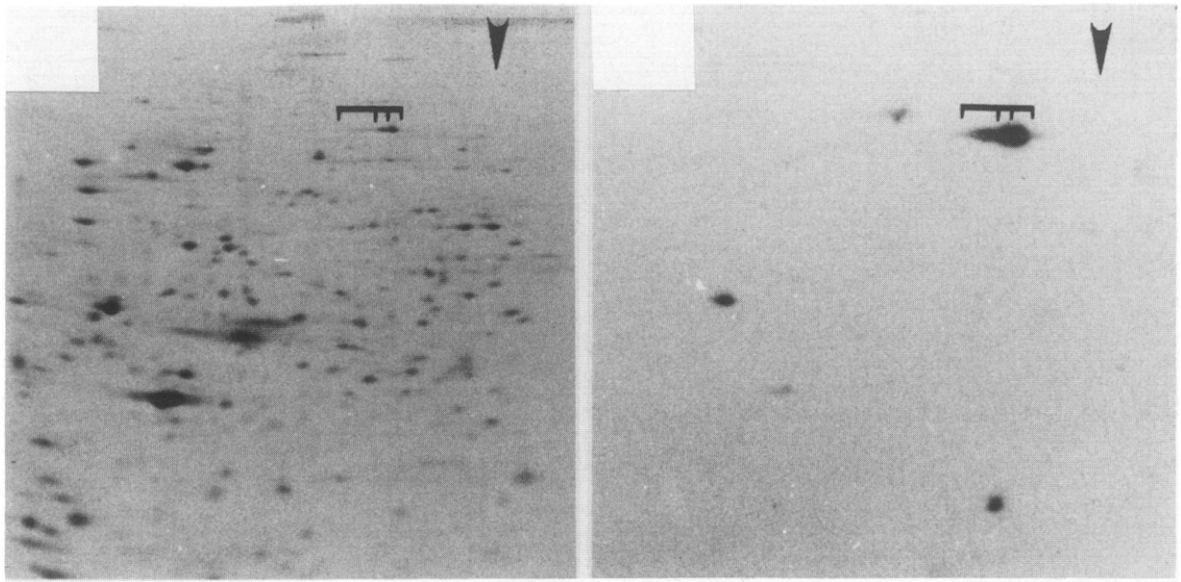

FIG. 6. Autoradiographs of 2-dimensional gel electrophoresis of extracts from human fibroblasts, labelled for 16 h with [^{35}S]methionine. (a) Total cell lysate; (b) vinculin immunoprecipitate. The arrowheads point to the location of chicken β vinculin which was mixed with the sample as an internal marker. The brackets indicate the 3 major human fibroblast isoforms (from right to left: γ, β and α).

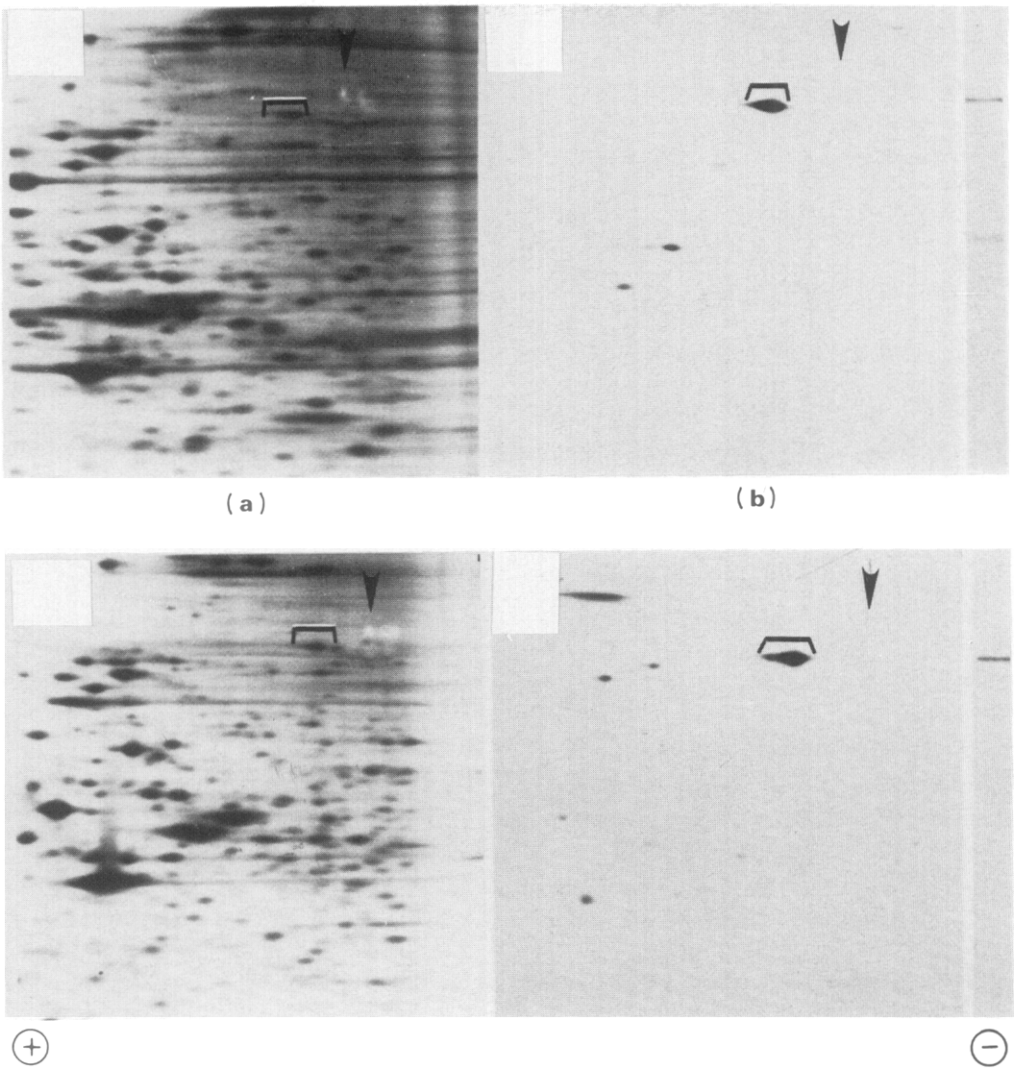

FIG. 7. Autoradiograms of 2-dimensional gel electrophoresis of total cell lysates ((a) and (c)) or vinculin immunoprecipitate ((b) and (d)). The cells, epidermal carcinoma A-431 ((a) and (b)) and neuroblastoma B-104 ((c) and (d)) were labelled with [^{35}S]methionine for 12 h prior to the extraction. The arrowheads mark the location of chicken β vinculin (as in Fig. 5), and the horizontal brackets point to the location of vinculins of the 2 cell lines. The columns at the right show the single-dimension electrophoresis of immunoprecipitated vinculin from A-431 cells (top) and from B-104 (bottom).

Coomassie blue staining of pure chicken gizzard vinculin marker, which was co-electrophoresed on the same gel (Fig. 8(a)). As indicated in the Figure, the entire ^{32}P-labelling in the vinculin region was found in an association with the acidic component of the α-vinculin region. Thus, only about 2 to 5% of the cellular vinculin exhibit a significant level of phosphorylation.

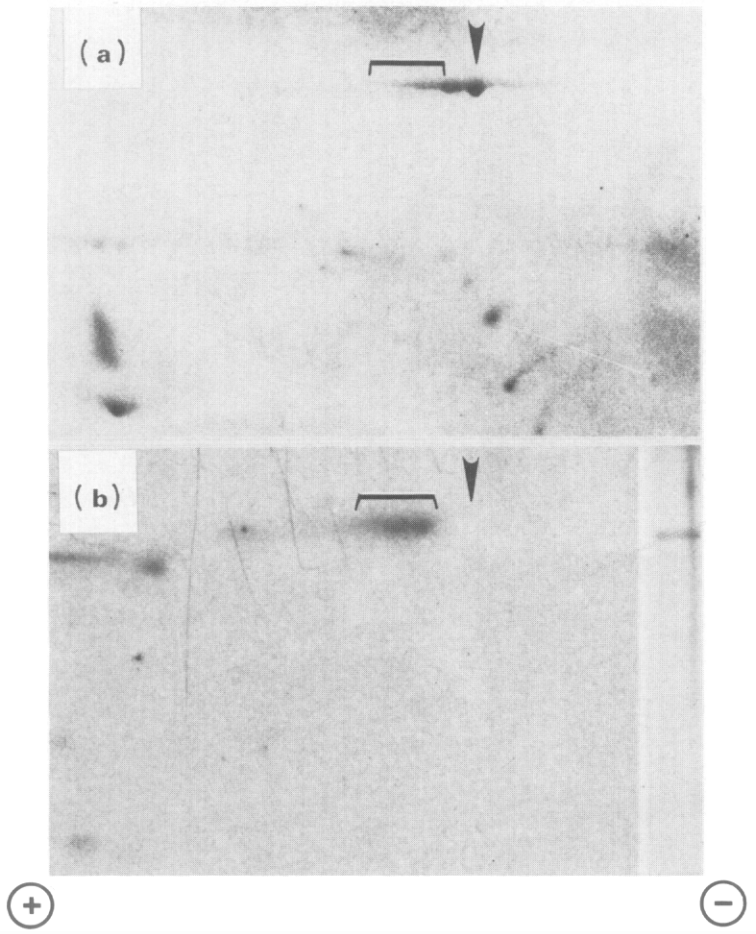

FIG. 8. (a) Coomassie blue staining and (b) autoradiogram of a 2-dimensional gel of ^{32}P-labelled vinculin. Chicken gizzard cells were incubated with 70 μCi [^{32}P]orthophosphate/ml for 12 h, then lysed, immunoprecipitated with vinculin antibodies and subjected to electrophoretic separation. The arrowhead points to the location of chicken β vinculin and the brackets mark the location of the phosphorylated vinculin. As may be seen, ^{32}P is apparently associated only with the α-vinculin isoform.

(f) *Spatial distribution of vinculin isoforms in cultured chicken cells*

As mentioned above, vinculin exists in cultured cells both as a cytoplasmic, easily extractable protein and in an organized, membrane- (and microfilament) bound form. The two pools of vinculin could be separated by selective extraction with detergent at pH 6·0. The immunofluorescent photomicrograph in Figure 9(a) shows a chicken gizzard cell that was fixed, permeabilized and immunolabelled for vinculin. It is apparent that, in addition to the membrane-bound vinculin, which was associated with focal contacts (arrows), significant amounts of the protein are diffusely distributed within the cell (see also Geiger, 1979). A short extraction with Triton X-100 removed the diffuse cytoplasmic vinculin and left mostly the

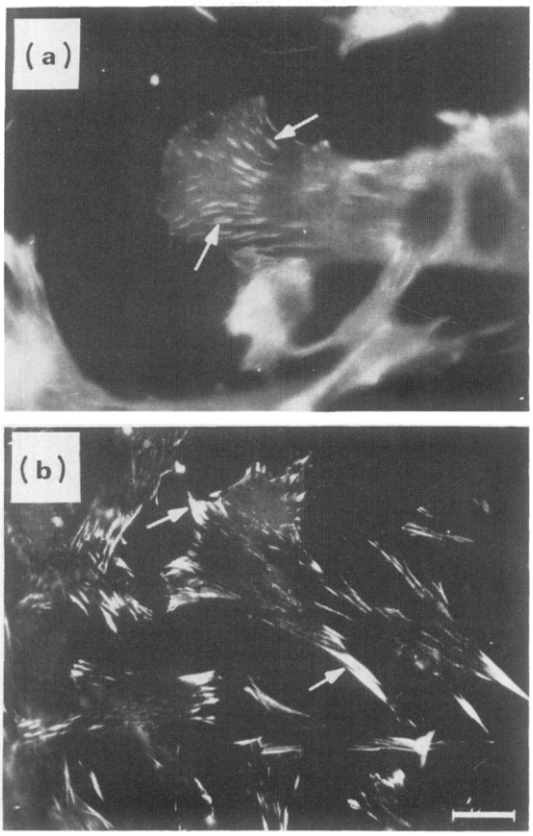

FIG. 9. Indirect immunofluorescent labelling of cultured chicken gizzard cells for vinculin using rabbit antibodies and rhodamine-labelled goat anti rabbit immunoglobulin G. (a) The cells were fixed first with formaldehyde, then permeabilized with Triton X-100 and labelled. (b) The cells were pre-extracted for 2 min with Triton X-100 at pH 6·0 prior to fixation, then fixed and immunolabelled. Notice the presence of "diffusely organized" vinculin in the prefixed cells and its removal from those cells that were extracted before fixation. The arrows point to areas of focal contact. The bar represents 10 μm.

membrane-bound fraction (Fig. 9(b)). To test the spatial distributions of the various isovinculins, [^{35}S]methionine-labelled cells were extracted with Triton X-100 and the vinculin isoform pattern determined in the solubilized extract and in the substrate-attached residue. The results are shown in Figure 10(a) and (b). It was determined by direct counting of the radioactivity in slices of the electrophoretic gels that the Triton-solubilized fraction contained mostly ($>90\%$) the basic vinculin spot, β. The residue (consisting of the membrane and cytoskeleton-bound vinculin) showed a marked enrichment in the α' and α isoforms, which reached values of 20 to 30% each. Similar results were obtained in five independent experiments. It thus appears that the different pools of cellular vinculin vary in their isoform composition, and that the acidic forms (which include the phosphorylated α) are enriched within the membrane-bound fraction.

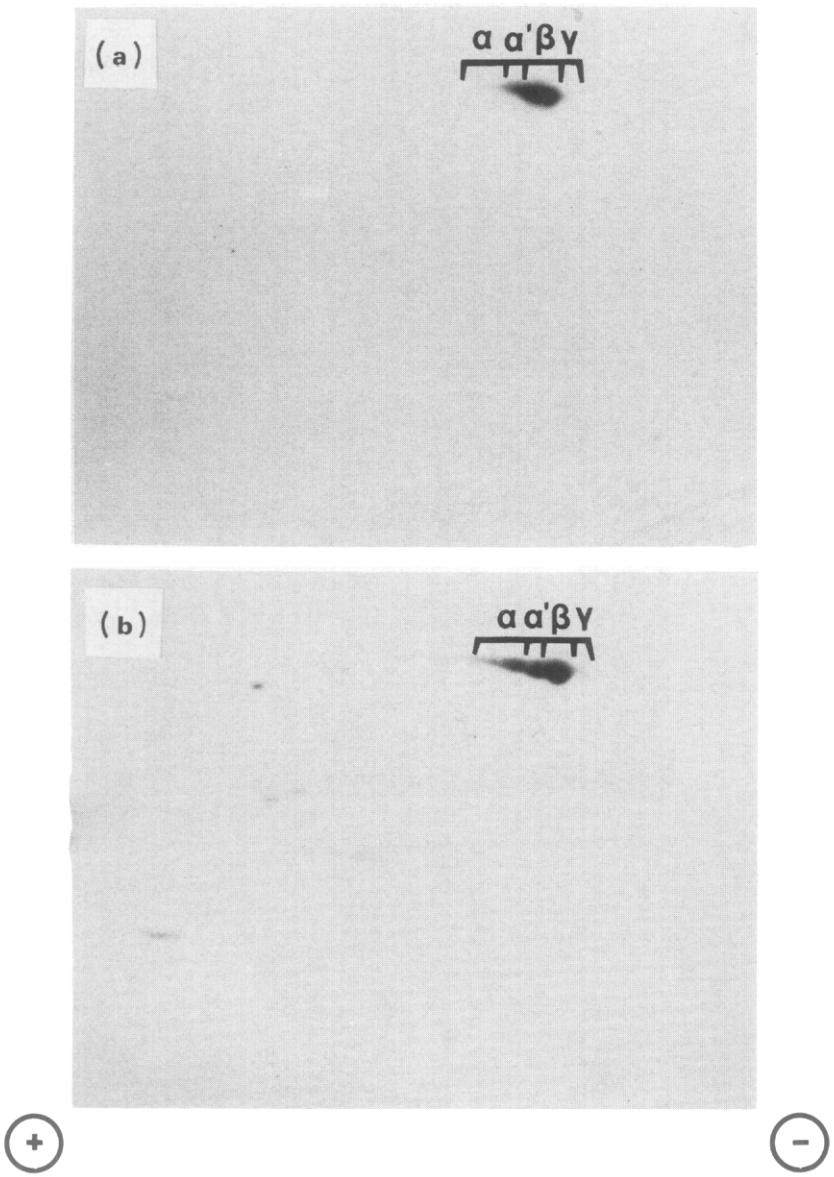

FIG. 10. Autoradiographs of 2-dimensional gel electrophoresis of immunoprecipitated vinculin from detergent-extracted chicken gizzard cells. The cultured cells were incubated with [^{35}S]methionine for 16 h, then extracted for 2 min with 0·5% (v/v) Triton as described in Materials and Methods. Both the soluble extract and the substrate-attached residue were immunoprecipitated with vinculin antibodies and subjected to isoelectric focusing. (a) Vinculin isoforms in the soluble Triton-extract. (b) Vinculin isoforms in the "organized" fraction of Triton-resistant vinculin. The brackets mark the locations of the major isovinculins: γ, β, α' and α. Notice that the soluble fraction contains predominantly β vinculin, while the residue is enriched with the α and α' isoforms.

4. Discussion

One of the most prominent events that follow the formation of cellular contacts is the transmembrane induction of microfilament organization. These relationships between the cellular contractile system and the focal contacts (or adhesion plaques) were pointed out by Abercrombie and co-workers (Abercrombie *et al.*, 1971; Abercrombie & Dunn, 1975) almost a decade ago and have been verified since by a variety of techniques, including electron microscopy, immunofluorescent labelling, interference-reflection analysis or a combination of these approaches (Izzard & Lochner, 1976,1980; Heath & Dunn, 1978; Wehland *et al.*, 1979). More recently, it was demonstrated that the protein vinculin is specifically associated with the areas of membrane–microfilament association in the contact areas (Geiger, 1979; Geiger *et al.*, 1980). In view of the close spatial relationships of vinculin and the cell membrane (Geiger *et al.*, 1981) and its ability to interact with actin (Jockusch & Isenberg, 1981), one may suggest that vinculin is involved in the linkage of actin to the membrane.

However, in addition to its association with focal contacts and streaks, vinculin may also be found in a soluble form in the cytoplasm. It was proposed that during cell spreading and locomotion, part of the soluble vinculin moves to the membrane and associates with it. To approach the possible molecular basis for the complex pattern of vinculin distribution, I have characterized here the heterogeneity of vinculin itself. The results described above indicate that vinculin in cells and tissues may exist in several closely related isoforms, which apparently exhibit different subcellular distributions.

The isovinculin pattern in different cells and tissues exhibited a considerable variability both in the number of isoforms and in their isoelectrophoretic range. Avian vinculins (from chicken or turkey) were more basic than mammalian vinculins (from human, mouse, pig and rat) by almost 0·8 pH unit. In chicken gizzard tissue, I could resolve five or six isovinculin spots, while the cell lines tested exhibited one to three vinculin polypeptides. A significant and reproducible difference was noted between the isovinculins of intact gizzard tissue and those detected in cultures of cells isolated from the same organ. Thus, the cultured gizzard cells did not express the γ-vinculin, which was one of the most prominent isovinculins of the intact tissue. The reason for this loss of expression of γ-isovinculin during cultivation *in vitro* is not clear. It is possible that the intercellular contacts in the tissue are necessary for the induction of γ-form formation or that the extensive artificial cell–substrate contacts of the cultured cells shut it off. Alternatively, the γ-vinculin may be produced by gizzard cells that are lost during cultivation. We have often observed, by immunolabelling with antibodies to muscle-specific intermediate filaments (desmin), that the ratio between smooth muscle cells (desmin positive) and fibroblasts of mesenchymal origin (desmin negative) may change during cultivation. These and other possibilities are being tested.

In spite of the marked charge differences between the more acidic and the more basic isoforms, several lines of evidence point to very close molecular interrelationships between them. Peptide map analysis of all the chicken gizzard

isovinculin spots (3 of which were shown here) pointed to a high degree of similarity. Similarly, the three major isovinculins of pig heart were closely related to each other and to the gizzard proteins. The remarkable interspecies homology found by the peptide map analysis suggests that vinculin and its isoforms are evolutionarily, highly conserved proteins. It should be pointed out, however, that the molecular properties of isovinculins described here and the comparison of their antigenic properties are still quite preliminary.

The heterogeneity of vinculin could, in principle, stem from either the co-ordinated expression of a gene family, from post-translational modifications or from a combination of the two.

Regardless of the molecular basis for the diversity of all the isovinculins, it was shown here that the protein may undergo phosphorylation with a strict isoform specificity. Thus, cultured chicken gizzard cells that were incubated with [^{32}P]orthophosphate incorporated the label preferentially into the α isovinculin. These results should be discussed in the light of the recent study by Sefton and co-workers of the phosphorylation of vinculin in Rous sarcoma virus-transformed cells (Sefton *et al.*, 1981). They have demonstrated that in uninfected cells, the specific incorporation of ^{32}P into vinculin was about 0·08 mol of phosphate per mol of protein under conditions of steady-state labelling. This value is similar to the relative content of α vinculin reported in this study. Since the phosphorylation of vinculin was associated almost exclusively with this isoform, it appears that the molar ratio of phosphate to protein (α vinculin) is about 1·0, and it is thus possible that α vinculin is formed by phosphorylation of one of the more basic forms. The present study reveals another specific property of the α (and perhaps also the α') isoform; namely, a unique association with the membrane or cytoskeleton-bound residue of Triton extraction. The cytoplasmic vinculin that could be extracted readily by the detergent consisted almost exclusively of the β form. The possibility that isoform interconversion occurred as a result of the extraction itself seems to be very unlikely; when we have mixed the "soluble" and the organized vinculins in the appropriate proportions and subjected them to two-dimensional gel electrophoresis, we have found exactly the same isoform pattern that was obtained with total cell extracts.

Is the phosphorylation of vinculin required for its transition from a soluble cytoplasmic state into a membrane-bound form, or does the phosphorylation occur after the association with the membrane is established? Moreover, is the process related to tyrosine-phosphorylation of vinculin by the p60^{src} in Rous sarcoma virus-transformed cells (Sefton *et al.*, 1981) and to the deterioration of the stress fibres and of the vinculin-containing focal contacts in these cells (David-Pfuety & Singer, 1980; Wang & Goldberg, 1976; Ash *et al.*, 1976; Edelman & Yahara, 1976)?

It is conceivable that vinculin is involved in several types of molecular interactions. It can bind to F-actin, as shown in studies *in vitro* (Jockush & Isenberg, 1981). It was also proposed that vinculin can associate (directly or indirectly) with components of the cell membrane (Geiger, 1981). This actin-independent interaction with the cell membrane is supported by our recent studies (Avnur, Small & Geiger, unpublished results), which indicate that fragmin from *Physarum* may remove actin from isolated substrate-attached membranes without

significantly affecting vinculin distribution. It still remains to be determined whether isoform diversity is related to the different functional interactions of vinculin.

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