Vinculin Interaction with Permeabilized Cells: Disruption and Reconstitution of a Binding Site

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Abstract. Fluorescently labeled vinculin binds to focal contact areas in permeabilized cells independent of actin (Avnur, Z., J. V. Small, and B. Geiger, 1983, J. Cell Biol., 96:1622–1630), but the nature of the binding site is unknown. In this study we have examined the interaction of vinculin with these sites in permeabilized L6 myoblasts to define conditions that perturb the binding and subsequently to reconstitute it. Mild treatment with low concentrations of protease prevents vinculin incorporation without gross changes in the cytoskeleton or extensive protein breakdown. Exposure to buffers of moderate ionic strength also reduces subsequent vinculin binding without large morphological effects. These extraction conditions were used to obtain a fraction from gizzard which was able to restore the vinculin localization. Talin, actin, and vinculin itself were able to alter the binding of labeled vinculin to permeabilized cells and each also interacted with vinculin in gel overlays; however, they were unable to reconstitute the binding site in treated permeabilized cells. The results show a requirement for an as yet unidentified protein to capacitate vinculin binding to focal contact sites and suggest that this protein is peripheral and interacts directly with the binding site.

VINCULIN is a soluble 130-kD protein which is concentrated at some, though not all, sites of microfilament–membrane interaction (13, 17); in particular in the focal contacts of cultured cells. These sites and the role of vinculin in them are of interest for several reasons: focal contacts are the principal sites of cell-substrate adherence and they are located at the termini of stress fibers, representing zones of microfilament anchorage to the plasma membrane (for review see references 4, 16, 26). Located as it is at the junction of the cytoskeleton, plasma membrane, and extracellular environment, the focal contact is an attractive candidate for a primary target of agents that result in morphological, adhesive, and cytoskeletal changes. In fact, the number and distribution of focal contacts is altered after transformation by some viruses (3, 10) or after treatment with tumor promoters (31). Further, vinculin is subject to phosphorylation by the viral kinase pp60src (20, 32) and by protein kinase C (the tumor promoter receptor) (34, 35); however, the significance of these events remains unclear in the absence of an understanding of vinculin function.

Although a number of proteins have been identified as components of the contact, very little is known of their arrangement and interactions (26). The presence of proteins responsible for linking the microfilaments to the plasma membrane would be expected; these would be functionally analogous to the spectrin–ankyrin–band 3 complex which binds actin to the membrane in erythrocytes (18). Vinculin is the best candidate (among identified focal contact proteins) for one such protein; interactions with filamentous actin (22, 36), talin (9, 28) (another focal contact protein), and vinculin itself (28) have been reported, although some of the apparent binding may have been due to impurities (11, 30). Other focal contact proteins are probably primarily concerned with actin bundling (alpha-actinin, fimbrin) or have unknown binding properties (e.g., FC-1 [27], 200-kD [25], and 130-kD [29] proteins). Talin does not interact directly with actin (8). Further information concerning protein–protein interactions in the focal contact is required in order to understand its structure and regulation.

Our approach to this problem has involved an examination of the binding of fluorescently labeled vinculin to residual focal contact areas in cultured cells permeabilized with non-ionic detergent. Geiger (15) first showed that labeled vinculin binds in an apparently natural fashion to the exposed inner surface of ventral membranes after most of the cell is removed. In combination with actin depolymerizing proteins, this technique has been used to demonstrate that the vinculin binding is independent of actin (1). The objective of the experiments reported here was to characterize the nature of the vinculin binding site and determine the possible role of proteins known to interact with vinculin in vitro. We describe procedures to disrupt the vinculin binding site without gross morphological alterations, and subsequently reconstitute it with extracts of smooth muscle. The results suggest that a heretofore undescribed protein is required for the vinculin binding.
Materials and Methods

Materials

The following reagents were used: tetramethylrhodamine isothiocyanate (Research Organics, Inc., Cleveland, OH); N-(7-nitrobenz-2-oxa-1,3-dia- zol-4-yl)phallacidin (Molecular Probes Inc., Junction City, OR); iodo gen (Pierce Chemical Co., Rockford, IL); sodium 125I-iodide (New England Nuclear, Boston); elastase (Worthington Biochemical Corp., Freehold, NJ). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Toronto, Ontario) and were reagent grade.

Cell Culture

L6 myoblasts (38) were routinely grown in alpha-modified Eagle’s medium containing 10% horse serum and 50 mg/liter gentamycin. For binding experiments, cells were plated at 50,000 per well in 35-mm dishes containing 18-mm square coverslips, and used after 2 d of growth.

Proteins

Vinculin was purified from chicken gizzard essentially as described (11) except that a pH 4.8 precipitation step was used before chromatography on DEAE cellulose. Vinculin remained in the supernatant during this treatment. Talin was purified from gizzard by the method of Burridge and Connell (6). Purity greater than 90% was estimated. Tropomyosin was also purified from gizzard (7).

Iodinated vinculin was prepared by the iodogen procedure (12) to a specific activity of 2,000–4,000 cpm/ng and separated from unbound isotope on a molecular sieve column. Tetramethylrhodamine-labeled vinculin (R-vinculin) and tropomyosin were prepared as described (1) except for the use of the isothiocyanate derivative. The labeled proteins were stored in small aliquots at −80°C. R-vinculin with chromophore/protein ratios of 0.5–1 was used.

Antibodies to vinculin were induced in rabbits and affinity purified on columns of Sepharose-linked antigen.

Gel Overlays

After SDS PAGE gels were fixed and washed in 40% isopropanol, 10% acetic acid (four 1-h washes). They were then rinsed in distilled water, washed in a pH 6 buffer (50 mM 2-[N-morpholino]ethane sulfonic acid [pH 6], 5 mM MgCl2, 3 mM EGTA, 1 mg/ml BSA) four times for 1.5 h each and incubated with iodinated vinculin (2–4 × 106 cpm in 5 ml of the pH 6 buffer) for 12–15 h at 4°C. Excess free label was removed by four 2-h rinses in buffer before staining, destaining, and autoradiography.

R-Vinculin Binding

Cells were rinsed in cold phosphate-buffered saline (PBS) and permeabilized for 30 s in a Triton X-100-containing buffer (50 mM NaCl, 10 mM Hepes [pH 6.9], 3 mM MgCl2, 0.3 M sucrose, 0.5% Triton X-100 [2]). Coverslips were then rinsed twice in cold "binding buffer" (25 mM 2-[N-morpholino]ethane sulfonic acid [pH 6], 3 mM MgCl2, 1 mM EGTA) before incubation with R-vinculin (30–50 μg/ml) in binding buffer on ice for 15 min. After two further quick rinses in binding buffer, coverslips were fixed in 3% paraformaldehyde in PBS, mounted in 90% glycerol, 10% 1 M Tris-HCl (pH 8), and viewed with epifluorescent optics in a Zeiss microscope. Conditions for tropomyosin binding were identical except that the interaction was observable only over a fairly narrow pH range. No focal contact fluorescence in the nuclear area may represent protein trapped in the thickest region of the cell which is not removed during the short rinse period.

Preparation of a Reconstituting Fraction

All steps were done at 4°C. Fresh chicken gizzards were dissected free of fat and connective tissue and 150 g of the muscle ground in a meat grinder, then homogenized in 5 vol of chilled acetone with a Waring blender (20 s at low setting, 10 s at high). The homogenate was stirred for 5 min before straining through four layers of cheese cloth. Excess acetone was squeezed out and the remaining material homogenized as above in 5 vol of extraction buffer containing 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was stirred for 15 min then centrifuged at 10,000 g for 10 min. Solid ammonium sulfate was added to the supernatant to 30% saturation (16.4 g/100 ml) and stirred for 30 min before the precipitate was collected by centrifugation (15 min, 14,000 g). The pellet was resuspended in 40 ml of extraction buffer and dialyzed against the same buffer (3 × 20 vol) before clarifying by ultracentrifugation (200,000 g; 1.5 h). Samples remained active for at least 2 wk at 4°C or for a period of several months at −80°C.

Reconstitution Procedure

Permeabilized cells were treated to disrupt R-vinculin binding, and then rinsed twice in cold binding buffer and incubated with a mixture of R-vinculin and the reconstituting fraction (1:10 dilution) in binding buffer on ice for 15 min. Cells were then rinsed, fixed, and mounted as above.

Other Methods

To perform SDS PAGE, we used the buffer system of Laemmli (24) with a 6–15% polyacrylamide gradient. Protein measurements were done by the Hartree modification of the Lowry procedure (19).

Results

R-Vinculin Binding to Permeabilized Cells

As has been shown in several cell types (15), R-vinculin binds to discrete regions at the termini of stress fibers in the area of residual focal contacts as well as giving diffuse perinuclear fluorescence. This distribution closely parallels the pattern seen in fibroblasts fixed and stained with antibodies to vinculin (1, 15). Similarly, in permeabilized L6 myoblasts, R-vinculin is concentrated in regions at stress fiber termini (Fig. 1, a and c) and in the perinuclear area. The amorphous fluorescence in the nuclear area may represent protein trapped in the thickest region of the cell which is not removed during the short rinse period.

Optimal conditions for the patchy focal-type binding of R-vinculin included low ionic strength, a pH of 6, and the presence of magnesium or other divalent cation. At higher pH values (>7), very little staining of the focal contact region could be observed, whereas bright diffuse fluorescence predominated at lower pH (≤6). Thus the interaction was observable only over a fairly narrow pH range. No focal contact structures were seen in the absence of divalent cations; optimal binding required the presence of 2–10 mM magnesium. Among other divalent cations tested (all at 2 mM), calcium was as effective as magnesium, neither zinc nor copper could substitute, while manganese was partially effective. At pH 6, 25 mM NaCl markedly decreased the intensity of focal contact labeling and higher concentrations led to progressive further decrements, although faint binding was still observable at 200 mM. Binding was unaffected by prior treatment of the permeabilized cells with several protein modification reagents, including iodoacetamide (10 mM), diethylpyrocarbonate (5 mM), and acetone (5 min on ice); but it was, however, destroyed by formaldehyde fixation, raising some doubt about whether proteins were involved.

The effects of proteases on the binding, however, clearly

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1. Abbreviation used in this paper: R-vinculin, tetramethylrhodamine-labeled vinculin.
indicated the necessity of protein(s). After treatment of the cell residues with low levels of chymotrypsin or elastase, subsequent binding of R-vinculin in the focal contact area was reduced to undetectable levels (Fig. 1 b), although some perinuclear fluorescence remained. This effect was not due to proteolysis of the labeled vinculin by residual protease, since the R-vinculin used could subsequently bind to permeabilized cells in a normal pattern (not shown). Under these very mild conditions of proteolysis the appearance of the permeabilized cells remained unaltered by phase-contrast or differential interference contrast microscopy. The pattern of stress fibers also remained intact after protease treatment (compare Fig. 1 c and d). Stress fibers in treated cells appeared slightly less well ordered and discrete (Fig. 1 d), perhaps indicative of some damage. By SDS PAGE, little protein breakdown was evident (Fig. 2); only minor decreases in the intensity of some stained bands could be observed. Among proteins from the cellular residues, high molecular mass bands (>90 kD) were slightly reduced in intensity by the protease treatment (Fig. 2, lanes a and b). More low molecular mass protein was also released during incubation (Fig. 2, lanes c and d) although neither effect was striking. This is perhaps not surprising in view of the suboptimal pH and temperature (for the protease) used, and suggests that the protein or proteins affected contain highly protease-sensitive regions.

In an effort to determine some of the proteins which could be responsible for vinculin binding, the gel overlay assay of Otto (28) was applied, except that the optimal conditions for vinculin binding to permeabilized cells were used (pH 6), rather than the higher pH used by others (not shown). $^{125}$I-Vinculin bound to purified talin, actin, and vinculin and, in extracts of L6, to a protein of ~220 kD (probably talin). Labeled vinculin also showed apparently nonspecific binding to phosphorylase b and IgG heavy chain. With the exception of the latter interactions, the results are very similar to the findings of others (9, 28, 37).

The ability of the various proteins in an un-denatured state to compete with, or otherwise alter, the interaction of R-vinculin with permeabilized cells was examined by including an excess of the protein in question in the binding reaction. Only actin, vinculin itself, and talin altered R-vinculin binding. Fig. 3 shows the effects of talin, phosphorylase b, and excess unlabeled vinculin. Phosphorylase b (Fig. 3 c) was without apparent effect, while vinculin (Fig. 3 d) decreased the intensity of labeling although a focal contact-type pattern remained evident. Talin led to bright diffuse labeling throughout the cell (Fig. 3 b) and increased background fluorescence on the coverslip. Thus, only those proteins which exhibit an apparent in vivo association with vinculin are able to alter R-vinculin binding.

**Extraction and Reconstitution of the Binding Site**

With the objective of developing conditions to remove a protein or proteins involved in the localization of vinculin from permeabilized cells without alterations in other residual cytoskeletal components, various mild treatments were attempted. Exposure of the cell residues to moderate ionic strength (0.15 M NaCl) at a slightly alkaline pH resulted in greatly decreased binding (compare Fig. 4, a and b). There was very little labeling near the cell margins where the bright R-vinculin patches are most evident in the untreated cells. Diffuse staining near cell nuclei remained, however. These conditions had no apparent effect on the binding of tetramethylrhodamine-labeled tropomyosin (Fig. 4, c and d), indicating that the actin network was not grossly affected. The
d) in a similar manner. Very little fluorescence is visible be-
tween these are myosin heavy chain (200 kD),
beta-galactosidase (116 kD), phosphorylase b (92 kD), BSA (67 kD),
IgG heavy chain (50 kD), actin (43 kD), and immunoglobulin
light chain (25 kD).

In preparing a fraction of smooth muscle protein which
would reconstitute vinculin binding, advantage was taken of
properties of the protein deduced from its behavior in peri-
meabilized cell residues. Proteins extracted from acetone-
treated gizzard with 0.15 M NaCl were fractionated by am-
monium sulfate precipitation and tested for their ability to
cause the reappearance of vinculin binding in extracted cell
residues. The 0–30% ammonium sulfate fraction was active
in this manner (compare Fig. 5, a and b). Faint residual stain-
ing in linear patches is observed near the cell margin in the
extracted cells (Fig. 5 b); however, much brighter, more dis-
crete patches occur at the edges of the cell when the gizzard
fraction is included (Fig. 5 a). This fraction was also able to
reconstitute binding in protease-treated cells (Fig. 5, c and
d) in a similar manner. Very little fluorescence is visible be-
yond the perinuclear area in proteolyzed cells (Fig. 5 d) but
the pattern of linear patches is clearly seen when the protein
fraction is present (Fig. 5 e). R-vinculin binding was restored
to levels qualitatively similar to control cells in the areas of
the focal contacts. The activity of the fraction was destroyed
by prior treatment with heat (95°C, 5 min) or chymotrypsin.

To determine whether the reconstitution resulted from the
interaction of a component of the fraction either directly with
the R-vinculin or with the binding site in the permeabilized
cells, reconstitution with the fraction was attempted in the
absence of R-vinculin, followed by washing and then bind-
ing. Extracted cells treated in this manner again showed
bright patches of fluorescence at the cell margins (Fig. 5 e); the
corresponding control is illustrated in Fig. 5 f. Little
difference in pattern was evident between cells reconstituted
by the two protocols (compare Fig. 5 a and e). Interestingly,
neither talin, vinculin, nor actin were able to reconstitute the
binding when added using either reconstitution protocol.

A gel of the reconstituting fraction is shown in Fig. 6. The
major protein band co-migrates with actin—as might be ex-
pected given the acetone resistance of actin—but a large
number of other proteins are present.

**Discussion**

Our interest in the focal contact area of cultured cells lies in
discovering how the formation and dissolution of the struc-
ture are controlled. The protein vinculin is of particular note
in this regard because its phosphorylation state changes in re-
sponse to transformation (32) and tumor promoters (35) and
microinjection of antibodies to vinculin leads to the disrup-
tion of focal contacts (5). Thus vinculin may play a regula-
tory role in the contact. In order to delineate the sequence
of events leading to altered focal contacts (and perhaps
thereby to cytoskeletal and behavioral changes) an under-
standing of the architecture and interactions of proteins in the
structure is necessary.

The characterization of vinculin binding to focal contact
areas in cultured cells offers one route to defining compo-
nents of the structure, although the significance of the results
depends on how closely the in vitro interaction reflects that
occurring inside the living cell. Several observations suggest
that the stress fibers are not unduly affected by the permea-
bilization process when appropriate buffers are used. The
fibers retain many of their associated proteins (including vin-
culin [I] and talin) and a variety of microfilament proteins
will bind in apparently normal distributions (14, 33). In addi-
tion, stress fibers retain functional integrity as measured by
their ability to contract in the presence of calcium and ATP
(23). Thus binding to stress fibers probably reflects at least
some of the interactions occurring in vivo.

This is borne out by the concentration of R-vinculin visible
in areas at the ends of stress fibers in L6 cells. The pattern
obtained is strikingly similar to that observed after immu-
nofluorescent staining with anti–vinculin antibodies. The
interaction meets criteria of specificity: it is reduced by the
inclusion of excess unlabeled vinculin, and denatured vincu-
lin does not bind (not shown). As found by Geiger (15), the
pH and salt optima for binding are somewhat lower than the
values inside cells. This may be explained by the extraction
of a necessary component at higher pH and salt values (see
below).

Our initial experiments were aimed at determining the na-
ture of the component(s) in the focal contact that are required
for vinculin binding. Vinculin is known to interact with an-
ionic phospholipids (21), raising the possibility of a direct
Figure 3. Effects of unlabeled proteins on R-vinculin binding to permeabilized cells. Cells were incubated with R-vinculin alone (a), or with 1 mg/ml of talin (b), phosphorylase b (c), or vinculin (d). Bar, 20 µm.

Figure 4. Effect of treatment with extraction buffer on R-vinculin and R-tropomyosin binding. Permeabilized cells were treated with binding buffer (a and c) or extraction buffer (b and d). After 15 min on ice, cells were rinsed and incubated with R-vinculin (a and b) or tetramethylrhodamine-labeled tropomyosin (c and d) for a further 15 min. Arrows in b indicate cell margins where prominent R-vinculin binding occurs in untreated cells. Bars, 20 µm.
Figure 5. Restoration of R vinculin binding by a gizzard fraction. Permeabilized cells treated with extraction buffer (a, b, e, and f) or with elastase (c and d) were incubated with R vinculin alone (b and d) or in the presence of a gizzard fraction (a and c). Cells in e and f were treated with the gizzard fraction and rinsed before R vinculin binding. Bar, 20 μm.

binding to domains of membrane lipid. The lack of effect with several protein modifying and denaturing reagents (diethylpyrocarbonate, iodoacetamide, acetone) also pointed in this direction. The existence of a protein component in the binding site is indicated, however, by the effects of protease treatment. Very mild treatment with either elastase or chymotrypsin results in the complete loss of subsequent R vinculin binding without disrupting the stress fibers morphologically or causing gross protein breakdown (Figs. 1 and 2). This effect indicates that a necessary protein has very sensitive
proteolytic cleavage sites and is either destroyed or released from the contact site. This protein could be directly involved in vinculin binding or act in some indirect fashion to capacitate binding.

Several proteins are known to interact with vinculin in vitro. The 215-kD protein talin binds vinculin with high affinity in several types of assay (9, 28, 37). A gel overlay method also indicates vinculin interactions with actin and with vinculin itself (as well as other proteins) (28). In these reports, however, the binding was performed at pH 7.5: a pH where very little interaction of R-vinculin with permeabilized cells occurs. The gel overlay method was therefore used to compare the interactions at the lower pH and to survey the binding proteins present in myoblasts. Purified vinculin, talin, and actin all retained the labeled vinculin, and the major binding protein of the L6 cell line had an Mr, very close to that of talin. In addition, we found that phosphorylase b and IgG heavy chain retained significant amounts of the probe. While interactions of vinculin with proteins found in the focal contact might be expected, binding to the latter proteins is very unlikely to occur in vivo and probably represents abnormal interactions with sites exposed by denaturation. In fact, neither phosphorylase b nor IgG in their native state was able to interfere with R-vinculin binding to permeabilized cells. By these methods, therefore, the major candidates for a protein responsible for R-vinculin binding are talin and vinculin itself. The binding is known to be independent of actin (1).

The results of competition experiments in which the inclusion of excess vinculin or actin reduced R-vinculin binding, and in which talin caused an overall diffuse increase in binding, are indicative of an interaction between the native forms of these proteins and either R-vinculin or its binding site. The decrease in intensity brought about by vinculin is probably due to simple competition for limited binding sites. The effects of talin may result from the formation of a talin–vinculin complex which sticks to a variety of structures in the cell, obscuring any interaction at the contact. Clearly, the simple fact that these interactions occur does not necessarily indicate that they are primarily responsible or even essential for the concentration of R-vinculin in the contact as observed in the fluorescence microscope. A disruption of the binding site and its subsequent reconstitution by purified proteins is required in order to demonstrate the essential components of the site.

Two sets of mild conditions were found to result in loss of R-vinculin binding: the protease treatment at pH 6, and extraction in 0.15 M NaCl at pH 7.3. Neither of these treatments caused drastic alterations of the residual cytoskeleton, and both allowed the subsequent reappearance of apparently normal R-vinculin binding after exposure to protein fractions from gizzard. The isolation of a reconstituting fraction relied on the properties of the binding site inferred from its behavior in permeabilized cells: acetone resistance and solubility in 0.15 M NaCl were exploited to prepare the extract. The evidence indicates that the active constituent is a protein: it is sensitive to chymotrypsin and heat, and is non-dialyzable. In addition, it replaces a component which is also protease sensitive. In principle, such a protein might interact with R-vinculin, a site in the focal contact, or both. These possibilities were partially distinguished by the experiment illustrated in Fig. 5. Since reconstitution of the binding occurred even when the tissue fraction was removed before the binding step, the effect can not be solely on R-vinculin. The protein in the fraction must act at the binding site in the cell. The simplest interpretation of the results is that the protein binds to vinculin and to another component of the contact site, directly causing the localization of R-vinculin, however, other possibilities remain.

The already-identified candidates for a role in vinculin binding—talin and vinculin itself—were unable to reconstitute in this assay. Preliminary experiments have also indicated that vinculin and talin are not present in partially purified active fractions. For these reasons it is likely that the active component represents a hitherto unidentified component or mixture of components of the focal contact. Its solubility in buffers without detergents suggests a peripheral, rather than integral location in the membrane. One can speculate that vinculin interacts with talin, actin, and the protein to help form a dense focal contact structure stabilized by multiple protein–protein contacts.

Our use of permeabilized cells and fluorescently labeled protein to define some of the characteristics of binding and to dissect the binding site is of potential utility to other structural proteins. Clearly, binding in a recognizable pattern to a structure which is stable through the permeabilization procedure must occur for this type of approach to be successful. Discovering conditions which specifically disrupt or remove parts of the binding site while still allowing reconstitution is an empirical process, however, manipulation of ionic concentration and the use of proteases has been successful in the present case as well as in the discovery of the spectrin–binding protein ankyrin (for review see reference 18). This approach suffers from the qualitative nature of the data obtained and the tediousness of the assay, but it allows the examination of
binding sites in a controlled ionic environment in situ or ganelles that are difficult to isolate. Two important points arise from our application of the technique; vinculin binding involves at least one protein, and this protein is different from talin or vinculin. Further characterization of the activity in the tissue extracts may lead to the identification of the unknown factor and clarification of the role of vinculin in the focal contact site.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. E. H. Ball is a recipient of a Medical Research Council of Canada scholarship; C. Freitag was partially supported by an Ontario Graduate Studentship.

Received for publication 14 November 1986, and in revised form 11 April 1986.

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