Identification of Two Distinct Functional Domains on Vinculin Involved in Its Association with Focal Contacts

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Abstract. We report here on the identification of two distinct functional domains on chicken vinculin molecule, which can, independently, mediate its interaction with focal contacts in living cells. These findings were obtained by immunofluorescent labeling of COS cells transfected with a series of chicken vinculin-specific cDNA constructs derived from clones cVin1 and cVin5 (Bendori, R., D. Salomon, and B. Geiger. 1987. *EMBO [Eur. Mol. Biol. Organ.] J.* 6:2897–2905). These included a chimeric construct consisting of 5′ sequences of cVin1 attached to the complementary 3′ region of cVin5, as well as several constructs of either cVin1 or cVin5 from which 3′ or 5′ sequences were deleted. We show here that the products of both cVin1 and cVin5, and of the cVin1/cVin5 chimera, readily associated with focal contacts in transfected COS cells. Furthermore, 78 and 45 kD NH_2-terminal fragments encoded by a deleted cVin1 and the 78-kD COOH-terminal portion of vinculin encoded by cVin5 were capable of binding specifically to focal contact areas. In contrast 3′-deletion mutants prepared from clone cVin5 and a 5′-deletion mutant of cVin1, lacking both NH_2- and COOH-terminal sequences, failed to associate with focal contacts in transfected cells. The loss of binding was accompanied by an overall disarray of the microfilament system. These results, together with previous in vitro binding studies, suggest that vinculin contains at least two independent sites for binding to focal contacts; the NH_2-terminal domain may contain the talin binding site while the COOH-terminal domain may mediate vinculin–vinculin interaction. Moreover, the disruptive effect of the double-deleted molecule (lacking the two focal-contact binding sites) on the organization of actin suggests that a distinct region involved in the binding of vinculin to the microfilament system is present in the NH_2-terminal 45-kD region of the molecule.

ADHERENS junctions (AJ)[1] are a specialized class of cell contacts, formed between neighboring cells or between cells and the extracellular matrix. They are characterized by their association with actin microfilament bundles at their cytoplasmic faces (Abercrombie et al., 1971; Geiger et al., 1983) and by the presence of vinculin-rich submembrane plaques (Geiger, 1979, 1983; Geiger et al., 1985*a*,*b*; Burridge, 1986). Although all adherens junctions apparently share a common feature of association with vinculin and actin, they also exhibit a considerable molecular heterogeneity: it has been shown that the cell–cell AJ contain specific integral membrane components such as A-CAM (Volk and Geiger, 1984, 1986*a*,*b*), uvomorulin (Boller et al., 1985), and a cytoplasmic plaque protein, plakoglobin (Cowin et al., 1986), which are not found in cell-matrix contacts. The latter, on the other hand, contain talin (Burridge and Connell, 1983*a*,*b*) and integrin (Buck and Horwitz, 1987; Chen et al., 1985; Damsky et al., 1985) which are apparently absent from intercellular junctions (Geiger et al., 1985).

Immunocytochemical localization of vinculin in a large variety of cultured cells and tissues have established its ubiquitous association with AJ (for review see Geiger, 1983, and Burridge, 1986). Moreover, fluorophore-conjugated chicken vinculin, microinjected into a variety of cells (Burridge and Feramisco, 1980; Kreis et al., 1984), or added to substrate-attached ventral membranes or to permeabilized cells (Avnur et al., 1983; Ball et al., 1986) was shown to bind specifically and avidly to focal contacts.

Biochemical studies have revealed that several electrophoretic isoforms of vinculin may be expressed in different cells (Geiger, 1982) and that muscle cells contain a higher molecular weight variant, namely metavinculin (Siliciano and Craig, 1982, 1987; Feramisco et al., 1982; Gimona et al., 1987, 1988*a*,*b*; Belkin and Koteliansky, 1987). In vitro binding studies have shown that vinculin may bind to other focal contact molecules including talin (Otto, 1983; Burridge and Mangeat, 1984) and possibly to α-actinin (Wilkins et al., 1983; Belkin and Koteliansky, 1987; Waschsstock et al., 1987). Moreover, electron microscopic analysis has demonstrated that avian vinculin consists of a globular head region and a rod-shaped tail. Examination of concentrated vinculin

1. *Abbreviation used in this paper*: AJ, adherens junctions.

solutions indicated that the molecules tend to self-aggregate, mainly through their tail domain (Milam, 1985; Molony and Burridge, 1985).

In spite of the wealth of information on the in vitro properties of vinculin and its distribution very little is presently known on its precise mode of interaction with the various junctional and cytoskeletal components in living cells. Biochemical studies have suggested a possible chain of transmembrane intermolecular interactions in the cell matrix AJ. These include transmembrane binding of integrins both to extracellular matrix (ECM) constituents and to talin (Horwitz et al., 1986), talin binding to vinculin (Otto, 1983; Burridge and Mangeat, 1984), and interaction of the latter with α-actinin (Wilkins et al., 1983; Belkin and Koteliansky, 1987; Wachsstock et al., 1987). This complex, once formed, may associate with F-actin filaments or nucleate their assembly (for discussion see Geiger et al., 1987). Though this hypothetical model accounts for many of the recent biochemical and immunocytochemical findings, the assignment of specific functions to the various components of AJ (Geiger et al., 1985) is still indirect and detailed information on the structure of the known junctional molecules and their functional domains is required.

To directly study the mode of interaction of vinculin with focal contacts in living cells we have recently isolated and characterized several chicken vinculin-specific cDNA clones (Bendori et al., 1987). Two of these clones, denoted cVinl and cVin5, were subsequently studied in detail. The 2.9-kb cVinl was found to be essentially identical to the cDNA isolated and sequenced by Price et al. (1987) coding for the NH_2-terminal head domain of chicken vinculin. The 4.9-kb cVin5 clone, containing the entire coding region of vinculin, was recently sequenced (Price et al., 1989), and was shown to be nearly identical, within the region of overlap, to that of the 2.89-kb clone of Price et al. (1987, 1989) except that the latter contained an additional 123-bp in-frame fragment absent from cVin5 as well as nine single base substitutions. Furthermore, examination of the protein product encoded by cVin5 indicated that it was devoid of talin binding capacity (Price et al., 1988, and Critchley, D., personal communication).

To locate functional domains involved in the association of vinculin with focal contacts, we have studied the expression of cVinl and cVin5 cDNA, transfected into COS cells. In addition, we prepared a series of 3'- and 5'-deletion mutants of both cDNA clones, transfected them into COS cells and studied the subcellular distribution of their products. We report here that the protein products of cVin5 and cVinl corresponding to the full-length vinculin and to the ~90-kD head domain, respectively, were both associated with focal contacts in the transfected cells. We further show that the 78- and 45-kD proteins encoded by 3'-deleted cVinl cDNA retained the ability to associate with focal contacts while similar 3'-deletions of cVin5 apparently inactivated the protein. It is further shown that deletion of the 45-kD NH_2-terminal fragment of cVinl abolished its capacity to bind to focal contacts, while similar deletion of cVin5 did not exhibit such an effect.

These results suggest that there are at least two focal contact binding sites located in distinct domains of the vinculin molecule. The implication of these findings on the molecular dynamics of junction formation is discussed.

Materials and Methods

Enzymes

All enzymes were purchased from New England Biolabs (Beverly, MA).

Immunochemical Reagents

Rabbit antibodies to chicken gizzard vinculin were prepared and affinity purified as previously described (Geiger, 1979). Goat anti-mouse Fab and goat anti-rabbit IgG were affinity purified on the respective immobilized antigens and iodinated by the chloramine T method (Hunter, 1973). Conjugation of lissamine-rhodamine B sulfonyl chloride to antibodies was carried out as described (Brandtzaeg, 1973). *N*[7-Nitrobenz-2-Dxa-1,3-diazol-4-yl]-phallacidine and rhodamine-conjugated phalloidine were purchased from Molecular Probes Inc. (Junction City, OR).

Most of the present study was carried out using rabbit antibodies specific for avian vinculin which do not react with the endogenous vinculin of COS cells. In addition, we have used a battery of mAbs prepared by T. Volk and T. Volberg in our laboratory, which showed distinct reactivities with vinculin and its fragments. To visualize the endogenous vinculin of COS cells we have used mAb vin 11.5 (now available from Sigma Chemical Co., St. Louis, MO), which reacts with both the avian and the mammalian proteins. Other monoclonal antibodies that were used to identify different regions of the chicken vinculin were as follows: (*a*) Antibodies reactive with the carboxy-terminal region of the head domain of vinculin and thus bind to the products of both intact cVinl and cVin5 (see below) are vin 9.3, vin 13.2, vin 7.4, and vin 11.5. (*b*) Antibodies recognizing a central portion of the vinculin molecule which is encoded by the region located between the Bam HI and the Pst I sites (see scheme in Fig. 1). These include mAbs vin 5.52, vin 3.32, and vin 14.6. (*c*) The polyclonal rabbit antibodies reacted with all these domains including the NH_2-terminal 45-kD portion of chicken vinculin encoded by the Eco RI–Bam HI sequence of both cVinl and cVin5.

Cells

COS cells, clone M6 (Gluzman, 1981), were kindly provided by Dr. M. Horowitz from our department. The cells were cultured in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS and maintained in a humidified incubator under an atmosphere of 8% CO_2.

Preparation of Constructs for Transfection

All transfections described here were carried out using the pSVL vector (Pharmacia Fine Chemicals, Uppsala, Sweden) into which the various vinculin cDNA clones were inserted. The fragments were ligated into the Sma I site of the pSVL polylinker.

To obtain the 2.2-kb Eco RI–Pst I and 1.4-kb Eco RI–Bam HI 5'-fragments of cVinl (see Fig. 1) or the 1.9-kb Eco RI–Pst I and 1.1-kb Eco RI–Bam HI 5' fragments of cVin5, the corresponding clones in pBR were double digested with the pBR respective restriction enzymes and blunted with either DNA polymerase I or with T4 DNA polymerase. The fragments were isolated from agarose gels and ligated into pSVL as above.

To obtain the 5'-deletion mutants (Bam HI–Eco RI) of cVinl and cVin5 (1.4 and 3.8 kb, respectively), each of the two cDNAs in pGEM-2 (Promega Biotec, Madison, WI) was cut out by Bam HI and ligated to the Bam HI site of pSVL. Correct translation of the Bam HI–Eco RI encoded mRNA was initiated at the first ATG located 3' to the Bam HI site at positions 1,480–1,482 of cVinl and 1,146–1,148 of cVin5.

Chimeric DNA construct consisting of Eco RI–Bam HI region of cVinl and the removal of Bam HI–Eco RI region of cVin5 (see Fig. 1) was prepared by removal of the 3' Bam HI–Eco RI region of cVinl in pSVL and inserting the 3' Bam HI–Eco RI end of cVin5 (3.8 kb).

Transformation was carried out in competent HB101 cells. Constructs displaying correct orientation were selected following restriction endonuclease mapping.

DNA Transfection into COS Cells

Transfections were carried out by the DEAE-dextran procedure essentially as described by Sompayrac and Danna (1981) with slight modifications: Subconfluent COS cells growing on 30-mm dishes or on glass coverslips were incubated with 1-ml DME containing 50 mM Tris HCl, pH 7.3, 0.2 mg/ml DEAE-dextran (mol wt 2×10^6; Pharmacia Fine Chemicals) and

10 μg DNA. After 10 h cells were extensively washed and replenished with DME containing 10% FCS. At 48 h posttransfection cells were either metabolically labeled and extracted (see below) or fixed and immunofluorescently labeled.

Biosynthetic Cell Labeling and Immunoprecipitation

Cells were metabolically labeled for 2 h with 50 μCi/ml [^{35}S]methionine (Amersham International, Amersham, UK; 1,200 Ci/mmol) in methionine-free DME and extracted with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% [wt/vol] SDS, 1% [wt/vol] Na-deoxycholate, 1% [vol/vol] Triton X-100, pH 7.2) in the presence of protease inhibitors (0.5 TIU/ml aprotinin and 2 mM PMSF). Equal amounts of TCA precipitable counts were immunoprecipitated, as previously described (Bendori et al., 1987).

Immunoblotting Analysis

Immunoblotting was performed essentially according to Towbin et al. (1979). The protein bands were electroblotted onto nitrocellulose sheets (Schleicher & Schull, Dassel, W. Germany) in 50 mM Tris-glycine buffer containing 1 mM $MgCl_2$. The nitrocellulose sheets were then incubated in 10% low-fat milk in PBS supplemented with 0.05% Tween-20, and then incubated with antibodies for 2 h. The immunoblots were rinsed in the same buffer and then incubated with ^{125}I-labeled goat anti-rabbit IgG for 2 h. After extensive rinsing in the same solution, blots were exposed to x-ray film.

Immunofluorescent Labeling

Transfected COS cells were briefly rinsed in 50 mM 2-(*N*-morpholino) ethane sulfonic acid (MES) buffer, pH 6.0, permeabilized for 1–2 min with 0.5% Triton X-100 in the same buffer and fixed with 3% paraformaldehyde in PBS. Immunofluorescent labeling was carried out as described by Geiger (1979).

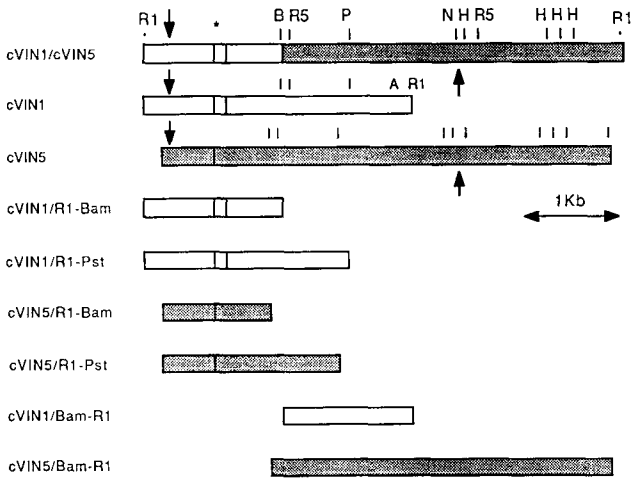

Figure 1. Schematic representation of the aligned vinculin-specific cDNA clones whose expression was studied in this work. All clones or regions derived from cVinl are shown as empty bars, all clones and regions derived from cVin5 are shaded. The segment indicated by the asterisk is the 123-bp segment found in cVinl and absent from cVin5 which is corresponding to the 41 amino acids (167–207). The arrows pointed up and down mark the initiation and termination sites, respectively. All the deletion mutants are designated according to the original clone from which they were derived and the restriction site at their 5′ and 3′ ends, respectively. The restriction sites (marked along the chimeric construct cVinl/cVin5) include Eco RI (*RI*), Bam HI (*B*), Eco R5 (*R5*), PST I (*P*), Nco I (*N*), Hind III (*H*). Note the extra AVa I site (*A*) present on cVinl exclusively. The Bam HI and Pst I sites were used to prepare the 3′ and 5′ deletion mutants.

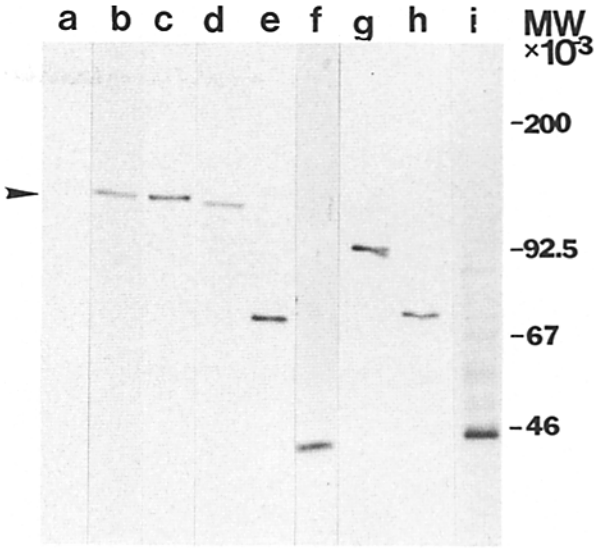

Figure 2. Immunoprecipitation of vinculin from metabolically labeled cells using rabbit antibodies specific for chicken vinculin. The cells examined included (lane *a*) nontransfected COS cells, (lane *b*) chicken embryo fibroblasts, and (lanes *c–i*) COS cells transfected with the various vinculin constructs: (lane *c*) the chimera of cVinl/cVin5; (lane *d*) cVin5; (lane *e*), cVin5/Eco RI–Pst I; (lane *f*) cVin5/Eco RI–Bam HI; (lane *g*) cVinl; (lane *h*) cVinl/Eco RI–Pst I; (lane *i*) cVinl/Eco RI–Bam HI. Note that the antibodies used here react only with the avian vinculin and its fragments. The apparent molecular weights of the various immunoreactive bands are: (lanes *b* and *c*) 116; (lane *d*) 112; (lane *e*) 75; (lane *f*) 42; (lane *g*) 93; (lane *h*) 78; and (lane *i*) 45.

Results

Transfection of COS Cells with the Chimera of cVinl/cVin5

As pointed out, sequence analysis of cVin5 and cVinl indicated that the two were nearly identical within the overlapping area, except that cVinl contained an extra fragment of 123 bp in frame, coding for amino acids 167–207, which were absent from cVin5 (Price et al., 1989) (see Fig. 1).

To study the expression and organization of full-length vinculin containing both the 41 amino acids at position 167–207 and the COOH-terminal "tail" we have transfected the chimeric DNA of cVinl/cVin5 into COS cells. Immunoprecipitation of metabolically labeled COS cells with chicken-specific vinculin antibodies 48 h posttransfection indicated that the chimeric cDNA encoded ~116-kD protein (Fig. 2, lane *c*) comigrating with chicken fibroblast vinculin (Fig. 2, lane *b*). The rabbit antibodies did not immunoprecipitate the endogenous vinculin of the COS cells in nontransfected cells (Fig. 2, lane *a*). The intrinsic COS vinculin could, however, be detected by immunofluorescent labeling with monoclonal antibody vin 11.5, which reacts with both avian and mammalian vinculin (see below).

Double immunofluorescent labeling of the cells transfected with the chimeric cDNA, using the chicken vinculin antibody and NBD-phallacidine, revealed a characteristic vinculin organization in the focal contacts of the cells (Fig. 3 *A*), associated with the termini of actin microfilament bundles

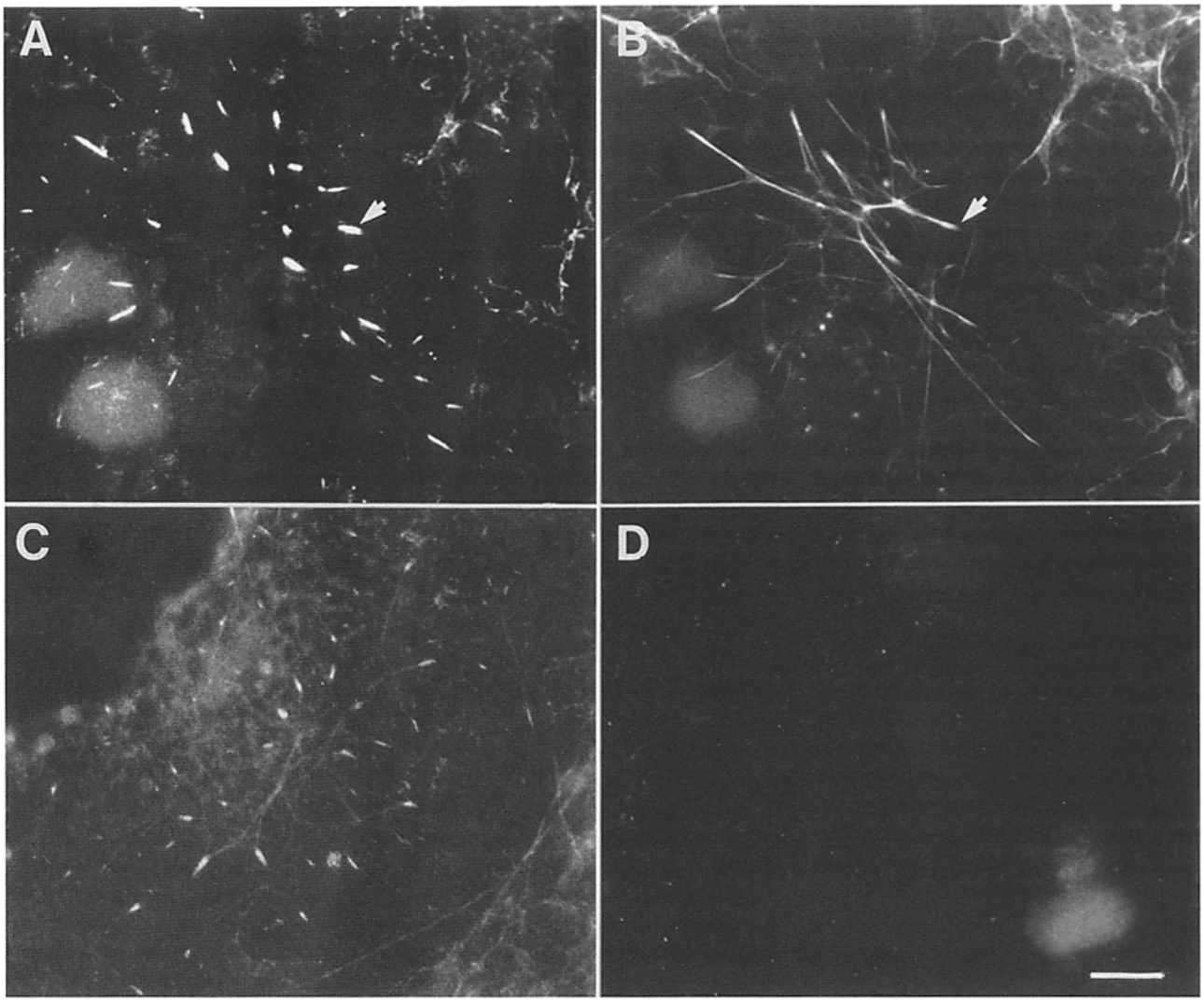

Figure 3. Organization of the chicken vinculin encoded by cVinl/cVin5 chimeric cDNA in COS cells. Double immunofluorescence labeling of transfected cells with rabbit anti-vinculin (*A*) and with NBD-phallacidine (*B*) showing specific association of the avian vinculin with focal contacts and the termini of actin bundles. Control, nontransfected COS cells labeled with the cross-reactive mAb vin 11.5 (*C*) or with the rabbit anti-chicken vinculin (*D*). Note that the distribution of the endogenous vinculin in nontransfected COS cells (*C*) is similar to that of the avian protein in transfected cells (*A*) and that the chicken-specific antibody does not react with the endogenous protein of the nontransfected COS cells (*D*). Bar, 10 μm.

(Fig. 3 *B*). Nontransfected COS cells were not labeled with the chicken vinculin antibodies (Fig. 3 *D*) while a characteristic array containing the endogenous focal contacts vinculin could be detected in these cells by using the interspecies cross reactive mAb vin 11.5 (Fig. 3 *C*).

Expression of cVinl and cVin5 cDNAs in COS Cells

To further characterize functional domains along the vinculin molecule which may mediate or affect its interaction with focal contacts, we have studied the pattern of expression and organization of clones cVin5 and cVinl. The former lacks 123 nucleotides encoding amino acid residues 167–207, while the latter encodes only 86% of the vinculin molecule lacking nearly the entire COOH-terminal "tail" domain.

Immunoprecipitation of metabolically labeled cVin5-transfected cells revealed that the encoded protein migrates close to, yet slightly faster than chicken fibroblast vinculin. This difference could be attributed to the absence of the 41 amino acids from the cVin5 sequence (Fig. 2, lane *d*). cVinl-transfected COS cells expressed a 93-kD protein as detected by SDS-PAGE (Fig. 2, lane *g*).

Immunofluorescent labeling of these cells revealed that the proteins encoded by both cVinl and cVin5 became associated with focal contacts in the transfected COS cells (Fig. 4 *A* and Fig. 5 *A*). Counter labeling of the same cultures for actin indicated that the focal contacts containing the exogenous chicken vinculin or vinculin fragments were invariably associated with actin bundles. Careful examination had indicated, however, that in addition to focal contact-associated labeling, the products of cVinl and cVin5 were often assembled into perinuclear filamentous or granular structures, as shown in Fig. 6.

Expression of 3′- and 5′-Deletion Mutants of cVinl and cVin5 in COS Cells

For the localization of functional domains which mediate or

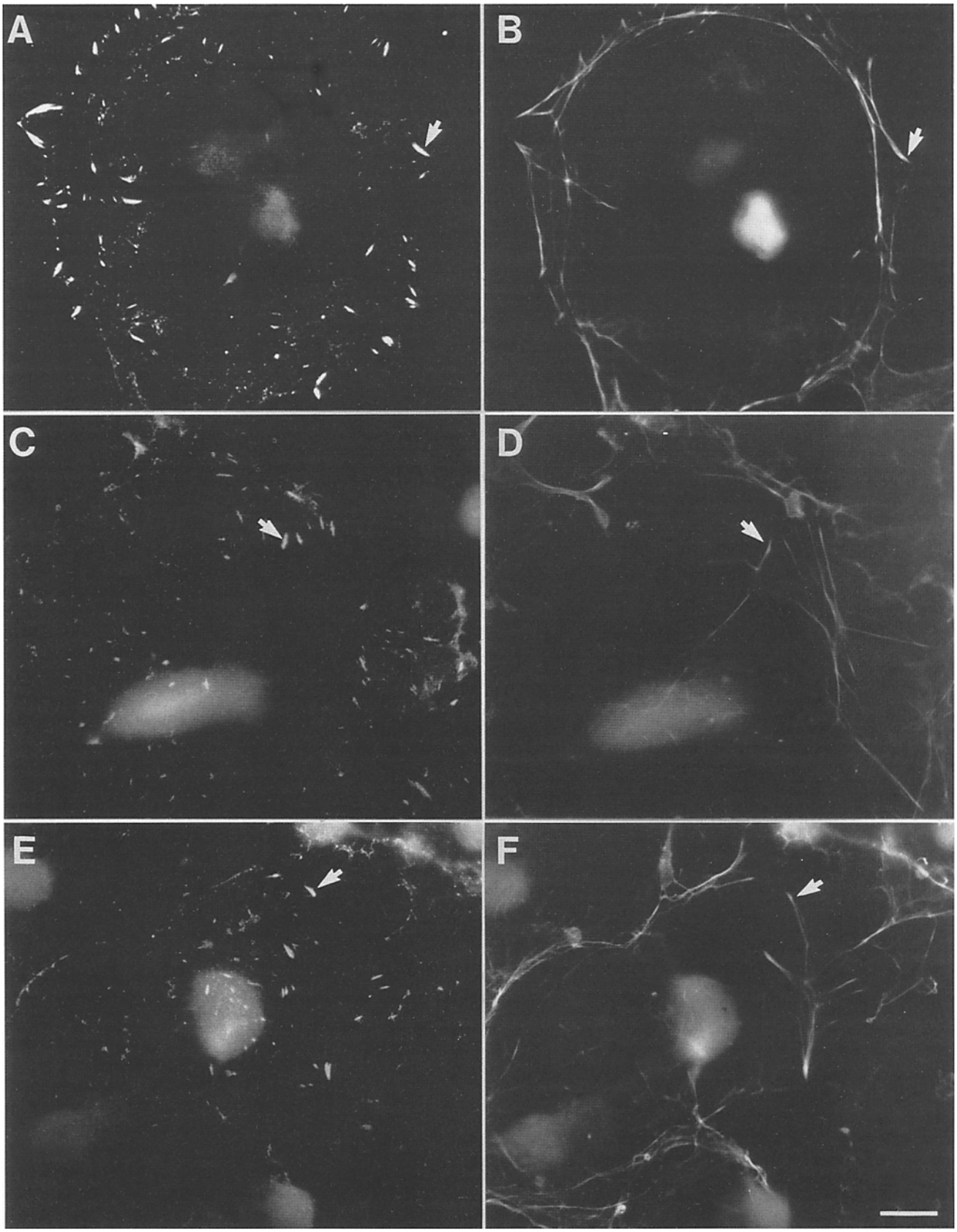

Figure 4. Organization of vinculin fragments encoded by cVinl and its 3′-deletion mutants in COS cells. Double-immunofluorescence labeling with chicken-specific rabbit anti-vinculin (*A*, *C*, and *E*) and with NBD-phallacidine (*B*, *D*, and *F*) of COS cells transfected with cVinl (*A* and *B*) with cVinl/Eco RI–Pst I, (*C* and *D*), or with cVinl/Eco RI–Bam HI (*E* and *F*). Note that the products of all constructs used bind to focal contacts and are associated with the termini of actin bundles (*arrows*). Bar, 10 μm.

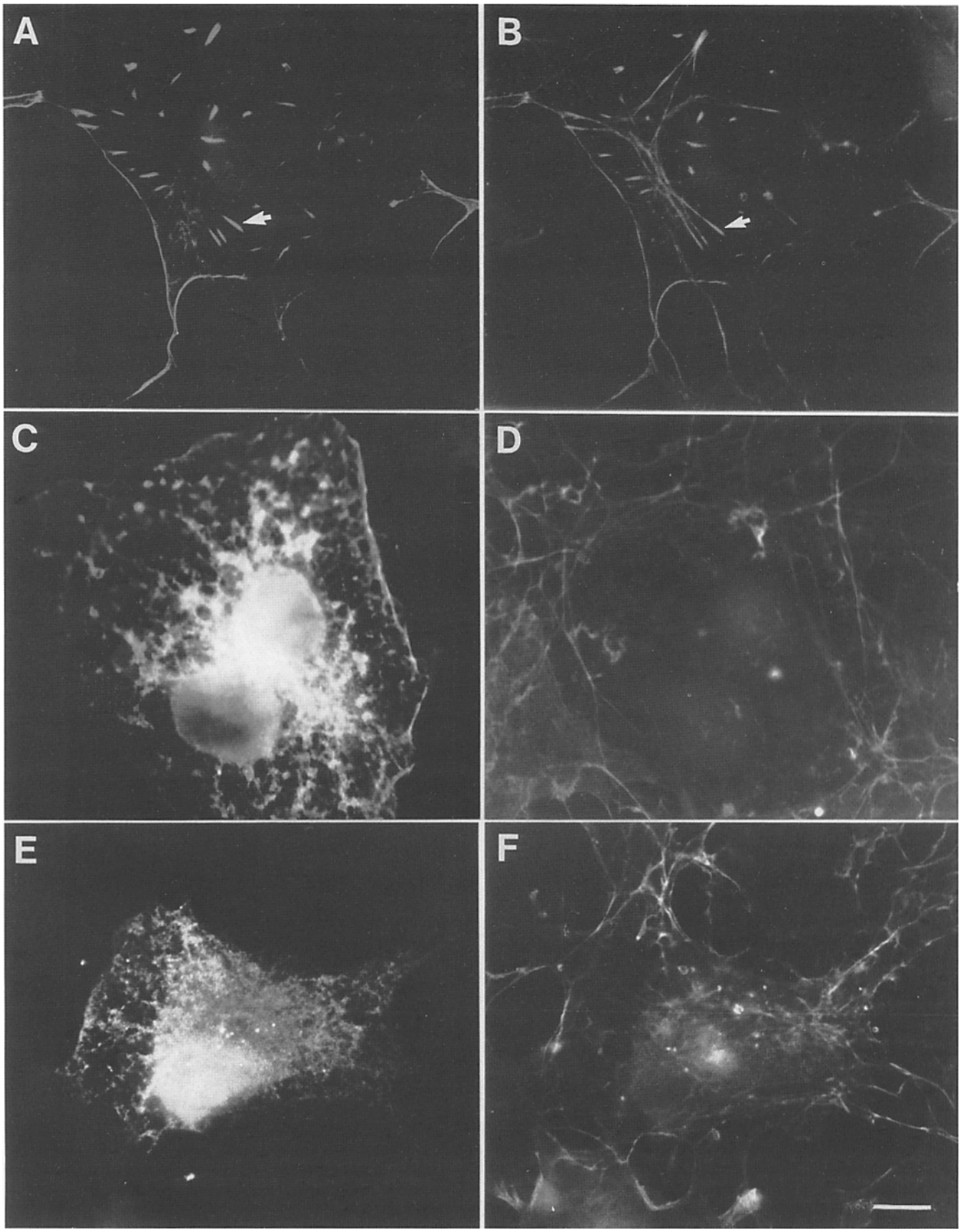

Figure 5. Organization of vinculin encoded by cVin5 and its 3′-deletion mutants in transfected COS cells. Double-immunofluorescence labeling with chicken-specific rabbit anti-vinculin (*A*, *C*, and *E*) and with NBD-phallacidine (*B*, *D*, and *F*). COS cells were transfected with the following contructs: cVin5 (*A* and *B*); cVin5/Eco RI–Pst I (*C* and *D*); and cVin5/Eco RI–Bam HI (*E* and *F*). Note that while the product of cVin5 associates with focal contacts both deleted vinculins lose the focal contact binding capacity. Actin in cells transfected with the latter constructs (*D* and *F*) appears disorganized. Bar, 10 μm.

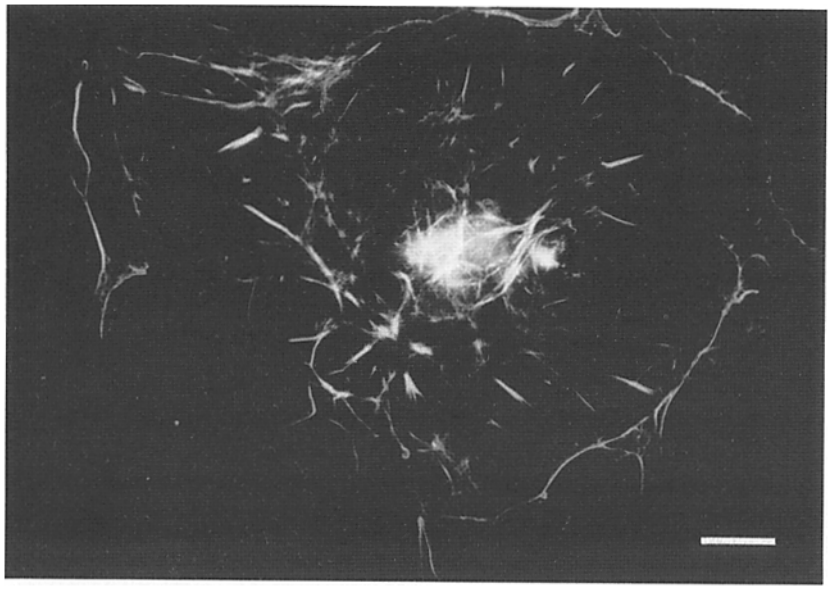

Figure 6. Immunofluorescence labeling of COS cells transfected with cVin5 using the chicken-specific rabbit anti-vinculin (as in Fig. 5 *A*). Note that in addition to the characteristic focal contact staining there is filamentous perinuclear staining. This pattern was observed in some of the transfected cells. Bar, 10 μm.

control the interaction of vinculin with focal contacts we have deleted 3′ sequences encoding segments of the carboxy terminal regions of cVinl and cVin5. Immunoprecipitation of metabolically labeled COS cells transfected with these cDNAs revealed a 78- and 45-kD immunoreactive polypeptides in cells transfected with cVinl/Eco RI–Pst I and cVinl/Eco RI–Bam HI (Fig. 2, lanes *h* and *i*), respectively. The corresponding deleted forms derived from cVin5 displayed lower molecular weights, in line with the absence of the 41 amino acids.

Immunofluorescent labeling of COS cells tranfected with the 3′-deletion mutants of cVinl pointed to a specific association of both the 78- and 45-kD segments of vinculin with focal contacts (Fig. 4, *C* and *E*). In all cases the pattern of labeling was similar to that of the intact cVinl and the labeled focal contacts were apparently associated with actin containing bundles (Fig. 4, *D* and *F*). This finding indicates that there are focal contact binding site(s) on the NH_2-terminal 45-kD segment of vinculin encoded by the Eco RI–Bam HI fragment of cVinl.

Similar deletion of 3′ segments from cVin5 cDNA either at the Pst I or the Bam HI sites resulted in the expression of 75 and 42 kD vinculin segments, respectively, in the transfected COS cells (Fig. 2, *e* and *f*). However, unlike the corresponding segments of cVinl and the intact cVin5, the proteins encoded by both 3′-deleted constructs of cVin5 (Fig. 5, *C* and *E*) completely lost the capacity to associate with focal contacts. Instead, these mutated proteins formed poorly defined meshes spread throughout the cytoplasm. These results suggested that there are two independent focal contact binding sites, the first is present on the NH_2-terminal 45-kD segment of the molecule and the other is located along the COOH-terminal tail (see Discussion below).

To supply a direct evidence for the presence of two distinct functional domains we have analyzed the expression and mode of organization of vinculin molecules, mutated at the NH_2-terminal area. We have prepared 5′-deletion mutants of both cVinl and cVin5 from which the 5′-Eco RI–Bam HI fragments (1.4 or 1.1 kb, respectively) were removed. The deletion of the 5′-segment of cVinl resulted in the expression of an immunoreactive 63-kD protein (Fig. 7, lane *b*) which formed an unorganized mesh throughout the cytoplasm of the tranfected cells (Fig. 8 *C*). In contrast, the 78-kD NH_2 terminus-deleted protein encoded by the Bam HI–Eco RI segment of cVin5 (Fig. 7, lane *a*) was organized in focal contacts (Fig. 8 *A*). It is noteworthy that the apparent molecular weights of the products of the 5′-deleted cVinl and cVin5 as

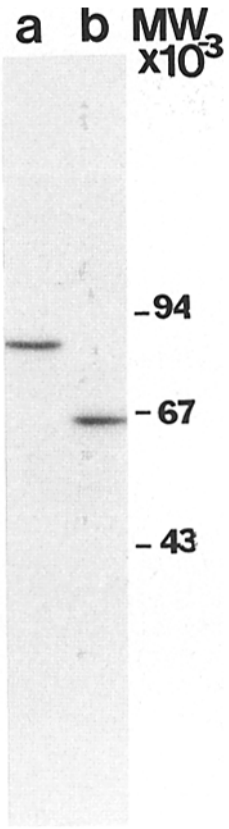

Figure 7. Immunoblotting analysis of vinculin fragments encoded by the 5′-deletion mutants of cVinl and cVin5 in transfected COS cells; COS cells transfected with cVin5/Bam HI–Eco RI (lane *a*) or with cVinl/Bam HI–Eco RI (lane *b*). The transfected cells were extracted, subjected to SDS-PAGE, electroblotted onto nitrocellulose, and reacted with chicken-specific rabbit anti-vinculin. The apparent molecular weights of the fragments are 78 (*a*) and 63 (*b*).

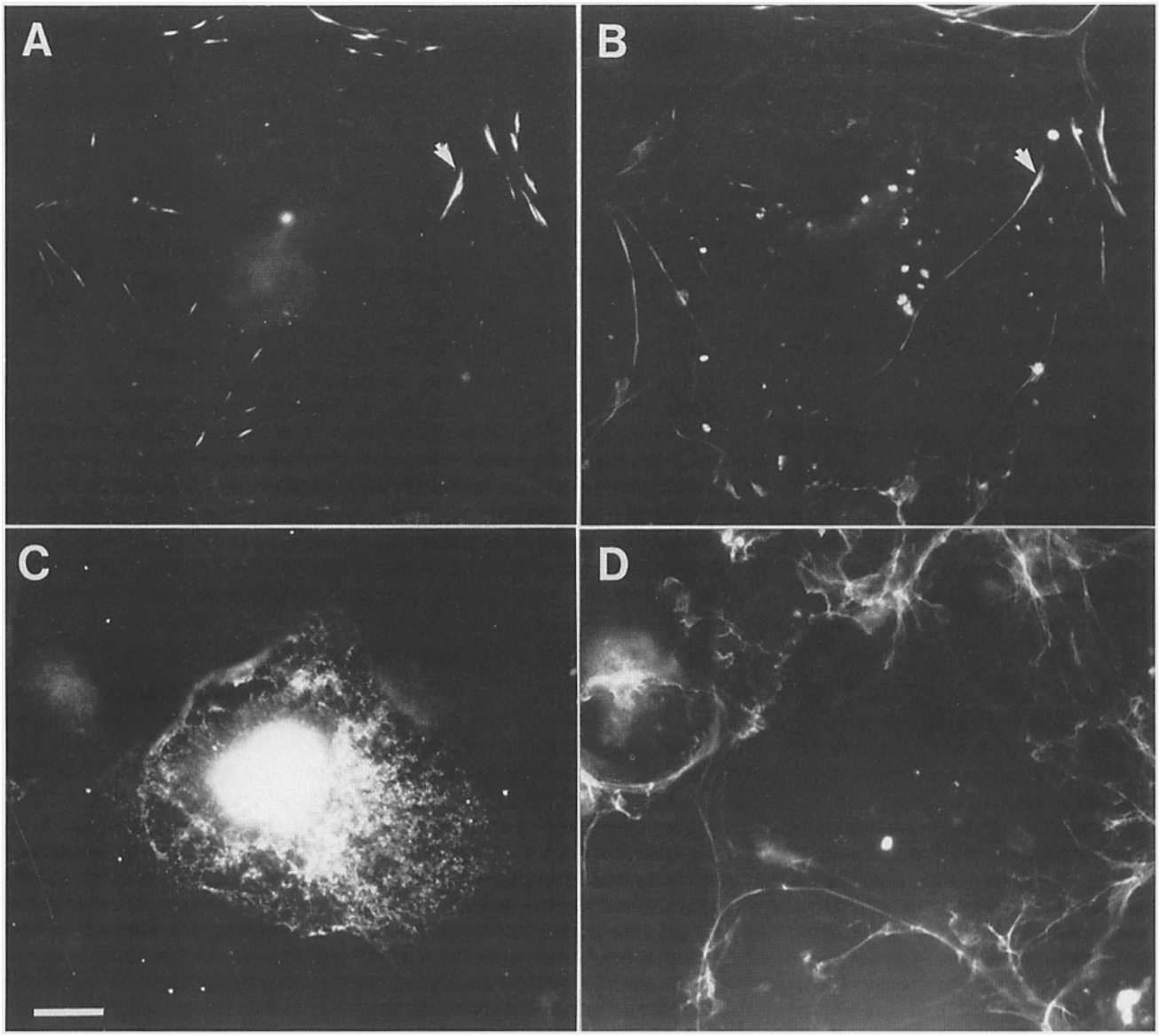

Figure 8. Organization of vinculin fragments encoded by the 5′-deletion mutants of cVinl and cVin5 in transfected COS cells. Double immunofluorescence labeling with chicken-specific rabbit anti-vinculin (*A* and *C*) and with NBD-phallacidine (*B* and *D*) of COS cells transfected with Bam HI–Eco RI fragment of either cVin5 (*A* and *B*) or of cVinl (*C* and *D*). Note that the deleted fragments of cVin5 retained the focal contact binding activity while the deleted fragments of cVinl lost this capacity. Bar, 10 μm.

determined by SDS-PAGE are 10–15% higher than the calculated values. The basis for these differences is not clear, but it may be related to the presence of a proline-rich region. These findings directly substantiate the notion that one focal contact binding site, is contained within the 45-kD NH_2-terminal region of vinculin and a second binding site is localized along the carboxy-terminal "tail" of the molecule.

Interestingly, examination of actin in COS cells transfected with all those constructs that fail to associate with focal contacts suggested that the organization of the endogenous microfilament system underwent significant deterioration. This may be appreciated from the double-labeled pairs in Fig. 5, *C–F* and Fig. 8, *C–D*.

Discussion

In the present study we have investigated the mode of association of vinculin with focal contacts in vivo by transfecting a series of intact and deleted chicken vinculin cDNA clones into living COS cells. Immunofluorescent localization of both the modified, exogenous avian vinculin, as well as the endogenous actin, enabled us to identify at least two distinct focal contact binding domains on vinculin and to point to another region involved in its interaction with the microfilament system. One binding site (site A) is present in the NH_2-terminal 45-kD region of the vinculin molecule and is most likely located at or depends on the presence of amino acid residues 167–207. The localization of site A is deduced from the fact that the 3′-deleted forms of cVinl (including the NH_2-terminal 45-kD fragment expressed by the cVinl/Eco RI–Bam HI clone) readily bind to focal contacts. This binding is abolished when the respective fragment does not contain residues 167–207 (cVin5/Eco RI–Bam HI). A second focal contact binding site (site B) appears to be located towards the COOH terminus of the vinculin molecule. This binding

domain is probably responsible for the association of the full-length cVin5 product with focal contacts. The exact location of site B is not clear, yet it seems likely that it resides within the tail domain of the molecule (see below). This is supported by the finding that deletion of the NH_2-terminal segment from cVin1 but not from cVin5 abolishes the capacity to react with focal contacts. The presence of another functional domain (site C) through which vinculin might interact with components of the microfilament system is indirectly suggested by the fact that exogenously expressed avian vinculin which lacks both focal contact binding sites A and B, exerts an apparent detrimental effect on the organization of the microfilament system of the transfected cells. It should nevertheless be emphasized that COS cells are probably not the most suitable host cells to approach this aspect since the endogenous microfilament system, even in nontransfected cells, is variable and often not highly elaborate. Attempts to address this aspect directly using cells which contain excessive network of stress fibers are now in progress.

To evaluate the results presented here it seems necessary to briefly consider the experimental system in which they were obtained, namely the transfection of avian vinculin cDNA into simian host cells and the immunocytochemical localization of the foreign proteins.

The capacity of the avian vinculin to bind to focal contacts of the COS cells was directly apparent from the transfection experiment shown here. Moreover, it has been previously shown that incubation of substrate-attached ventral membranes or detergent-permeabilized cells (both avian and mammalian) with rhodamine-labeled chick vinculin resulted in the specific binding of the latter to focal contacts, irrespective of the species of origin of the cells (Avnur et al., 1983; Ball et al., 1986). This interspecies interaction is not surprising in view of recent results indicating that vinculin is highly conserved throughout evolution and that avian and mammalian vinculin exhibit a considerable degree of sequence homology (Gimona et al., 1987, 1988*a*,*b*; Price et al., 1989). The interspecies differences in antigenicity have, however, enabled us to use chicken-specific vinculin antibody to selectively localize the foreign protein within the transfected cells. Furthermore, analysis of the immunofluorescent patterns suggests that the distribution of the foreign vinculin was essentially indistinguishable from that of the endogenous protein in nontransfected or mock-transfected cells, stained with the interspecies cross-reactive antibody vin 11.5 (see Fig. 3).

In the present study we have made no special effort to control the level of expression of the exogenous avian vinculin or to determine its levels relative to the endogenous protein. Such determination did not seem meaningful at this stage since the efficiency of the transient transfection was not completely uniform and since individual cells did display variable intensities of labeling. It should nevertheless be indicated that examination of cells at several intervals after transfection or cells which apparently express different levels of the exogenous vinculin did not suggest that these variations have a significant effect on the distribution of the protein. Attempts to directly approach the quantitative aspect are now in progress using permanent transfectants in which vinculin expression may be regulated by an inducible promoter.

The exact molecular nature and specificity of the interactions in which sites A, B, and possibly C of vinculin are involved have not been directly determined yet. However, taken together with previous data some suggestions as to the binding specificity of these sites may be made: It has been previously proposed that vinculin binds to several distinct molecules; these include talin, α-actinin, and additional vinculin molecules (Koteliansky et al., 1985; Otto, 1983; Wilkins, 1983; Avnur et al., 1983; Wachsstock et al., 1987). The association with talin, readily demonstrable by blot-binding assay (Otto, 1983; Burridge and Mangeat, 1984), occurs most probably via the NH_2-terminal head domain of vinculin since it was apparently fully retained by the major, 90-kD proteolytic fragment of vinculin (Milam, 1985; Molony and Burridge, 1985; Gimona et al., 1988*a*,*b*). Moreover, the talin binding activity of vinculin via "site A" most likely appears to reside in or depend on the 41 amino acids (residues 167–207) located within the head domain of vinculin (Price et al., 1989).

The presence of vinculin–vinculin interactions was suggested several years ago based on unexpected binding kinetics of rhodamine-labeled vinculin to focal contacts (Avnur et al., 1983). It was shown that the added vinculin associated with focal contacts, yet this binding was not readily saturable and could not be efficiently inhibited by the addition of excess unlabeled vinculin. It was thus proposed that vinculin might undergo self-association in focal contacts leading to a positively cooperative binding. This preliminary suggestion was supported by direct binding assays (Otto, 1983; Belkin and Koteliansky, 1987) and electron microscopic examination (Milam, 1985; Molony and Burridge, 1985). The latter analysis yielded especially interesting results since it suggested that vinculin molecules may form oligomeric aggregates, held together by their "tails" which are now known to reside in the sequences after the proline-rich region, towards the COOH terminus (Price et al., 1989). Based on these observations it is proposed that site B is a vinculin–vinculin binding site.

It is noteworthy that the presence of two apparently independent focal contact binding sites significantly affects the mode of vinculin association with focal contacts in living cells. It is conceivable that local immobilization or aggregation of the specific contact receptors present in focal contacts, most likely members of the integrin family (Tamkun et al., 1986), provides primary binding sites for talin (Horwitz et al., 1986; Burridge, 1986; Geiger et al., 1987; Buck and Horwitz, 1987) to which vinculin binds through site A. This immobilized vinculin may however bind through site B; additional vinculin molecules, which may bind more talin, etc., leading to the formation of a membrane-associated "plaque." The molecular nature of site C is somewhat less conclusive. The models depicting the possible molecular topology in AJ require the presence of some links between the talin and vinculin-containing plaque and the microfilament system. In vitro binding studies have pointed to the low-affinity, yet apparently specific binding of vinculin to α-actinin (Belkin and Koteliansky, 1987; Wilkins and Lin, 1983; Wachsstock et al., 1987). Such interaction is also in line with the enrichment of α-actinin, close to the cytoplasmic aspects of the submembrane plaques of AJ (Lazarides and Burridge, 1975; Geiger et al., 1980, 1981). The results presented here support the view that vinculin might be involved in these interactions, possibly via site C. This is based on the apparent capacity of vinculin variants which are devoid of both A and B focal contact binding sites to affect the overall integrity of

the microfilament system. These results also point to several open questions that may now be approached: It is still not clear what is the origin of the 41 amino acid difference noted between cVin1 and cVin5. In the present study this difference was effectively used for the demonstration of binding of vinculin through site B, yet protein or mRNA corresponding to cVin5 have not yet been unequivocally identified. Experiments addressing this aspect are now in progress.

A related issue concerns the molecular interactions of vinculin in intercellular AJ, which are apparently devoid of talin (Geiger et al., 1985). It is likely that further characterization of modified vinculins expressed in a variety of cells, using an approach similar to the one employed here, will shed light on the different molecular interactions and specific functional domains along the vinculin molecule and its particular roles in the assembly of AJ.

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