Adducin: Ca^{++}-dependent Association with Sites of Cell–Cell Contact

Hans W. Kaiser,* Edward O'Keefe,‡ and Vann Bennett*

*Howard Hughes Medical Institute and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710; and ‡Department of Medicine, University of North Carolina Medical School, Chapel Hill, North Carolina 27514

Abstract. Adducin is a protein recently purified from erythrocytes and brain that has properties in in vitro assays suggesting a role in assembly of a spectrin-actin lattice. This report describes the localization of adducin to plasma membranes of a variety of tissues and the discovery that adducin is concentrated at sites of cell–cell contact in the epithelial tissues where it is expressed. Adducin in tissues and cultured cells always was observed in association with spectrin and actin, although spectrin and actin were evident in the absence of adducin. In sections of intestinal epithelial cells spectrin was present on all plasma membrane surfaces while adducin was restricted to the lateral cell borders. Adducin also was not detected in association with actin stress fibers in cultured cells. The presence of adducin at cell–cell contact sites of cultured epithelial cells requires extracellular Ca^{++} and occurs within 15 min of addition of 0.3 mM Ca^{++}. Redistribution of adducin after addition of extracellular Ca^{++} is independent of formation of desmosomal and adherens junctions since assembly of adducin at contact sites requires lower concentrations of Ca^{++} and occurs more rapidly than redistribution of desmoplakin or vinculin. Treatment of keratinocytes and MDCK cells with nanomolar concentrations of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces redistribution of adducin away from contact sites. The effect of TPA may be a direct consequence of phosphorylation of adducin, since adducin is phosphorylated in TPA-treated cells and the phosphorylation of adducin occurs before disassembly of adducin from sites of cell–cell contact. Spectrin and adducin are both present in a detergent-insoluble form at cell–cell contact sites of cultured cells. These observations are consistent with the idea that adducin recognizes and associates with specific "receptors" localized at regions of cell–cell contact and promotes assembly of spectrin into a more stable structure, perhaps analogous to the highly organized spectrin-actin network of erythrocyte membranes.

PLASMA membranes of most eukaryotic cells contain a system of structural proteins organized around spectrin (fodrin) and referred to as the membrane skeleton (reviewed by Marchesi, 1985; Bennett, 1985; Goodman and Zagon, 1986). Spectrin is an elongated molecule, first characterized in erythrocytes, that associates at its ends with actin filaments and is attached to the plasma membrane by several linkages including association with ankyrin which in turn is linked to the anion transporter. Spectrin was initially localized in a uniform distribution on all surfaces of the plasma membrane (15, 20, 33), consistent with a simple structural role of supporting the lipid bilayer as is the case in erythrocyte membranes. A growing number of observations suggest that the spectrin-based membrane skeleton is more complex and diverse in its interactions than expected from the homogeneous distribution on plasma membranes. For example, a specialized isoform of ankyrin may have a role in maintenance of specialized membrane domains based on observations of in vitro association and colocalization with the Na/K ATPase in basolateral domains of distal tubule cells in kidney (27, 18) and with the voltage-dependent Na channel at nodes of Ranvier (37, 19). Additional evidence for a role of the spectrin skeleton in cell polarity is the observation that spectrin is present in a detergent-stable form concentrated at sites of cell–cell contact in cultured epithelial cells (25, 26).

Understanding the behavior of spectrin in cells will require a detailed knowledge of the proteins that interact with spectrin and define its localization and organization. Adducin is a protein recently purified from erythrocytes and brain (2, 12) that may play an important role in assembly of a spectrin–actin lattice (2, 13, 14, 24). Adducin, derived from adducere (bring together), promotes binding of spectrin to actin. Adducin binds preferentially to spectrin–actin complexes and recruits additional spectrin molecules to these complexes. Adducin is a calmodulin-binding protein (2, 12), and Ca^{++}/calmodulin inhibits the adducin-dependent recruitment of spectrin to spectrin–actin complexes (13). Adducin is phosphorylated by cAMP-dependent protein kinase and protein kinase C (5, 12, 32) although modifications of adducin activities through these modifications are not yet known. The activity of adducin is likely to have relevance beyond the erythrocyte and brain since polypeptides cross reacting with

adducin antibodies are associated with membranes of kidney, liver, lens, and lung (2).

In this article we describe the localization of adducin to plasma membranes of various rat tissues. Studies with cultured epithelial cells demonstrate the Ca^{++}-dependent redistribution of adducin to areas of cell–cell contact, a possible role for adducin in determining the organization of the spectrin-based membrane skeleton in these areas, and redistribution of adducin by nanomolar amounts of 12-*O*-tetradecananoylphorbol-13-acetate (TPA).

Materials and Methods

Reagents

Affinity-purified antibodies against brain adducin and brain spectrin were prepared from rabbit antisera as described (2, 7). Monoclonal antibodies against vinculin were from ICN Radiochemicals (Irvine, CA); monoclonal antibodies against desmoplakin I and II were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); and rhodamine-labeled phalloidin was from Molecular Probes Inc. (Junction City, OR). Rhodamine-labeled goat anti-rabbit IgG and fluorescein-labeled goat anti-mouse IgG were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Cell Culture

Keratinocytes were obtained from foreskins of newborn infants and initiated into culture by a modification (29) of the method of Rheinwald and Green (34) and then subcultured in MCDB medium 153 according to Boyce and Ham (3) with 0.1 mM Ca^{++}. Medium was supplemented with hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), EGF (5 ng/ml), and bovine pituitary extract (150 μg/ml) and with supplemental essential amino acids (histidine, 2×10^{-4} M; isoleucine, 7.5×10^{-4} M; methionine, 9×10^{-5} M; phenylalanine, 9×10^{-5} M; tryptophan, 4.5×10^{-5} M; and tyrosine, 7.5×10^{-5} M). Cells were subcultured at 5,000 cells/cm as previously described (31). Madin–Darby canine kidney (MDCK) cells were grown on plastic tissue culture plates in DME medium supplemented with 10% FCS. The cells were passaged twice weekly.

Immunofluorescence

Cells were cultured on coverslips for 3 d, fixed with 2% formaldehyde in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (PBS) for 30 min and extracted with 0.25% Triton X-100 in PBS for 5 min unless otherwise stated. The coverslips were incubated for 15 min with 2% BSA to block nonspecific binding. Antibody incubation was performed by overlaying each coverslip 14–16 h at 4°C with 40 μl of affinity purified IgG (~10 μg/ml in PBS). After a 30-min wash with PBS, coverslips were incubated for 90 min at 4°C with rhodamine-labeled goat anti-rabbit IgG diluted with PBS (1:40), and finally washed 30 min with PBS and mounted in 50% glycerol. For colocalization studies of different antigens in one sample double immunofluorescence was performed using both rhodamine and fluorescein as labels for antibodies. Basically the same protocol was used as described above but antibody incubation was performed by mixing antibodies from rabbit with antibodies from mouse. In the second labeling step a mixture rhodamine-labeled goat anti-rabbit IgG and fluorescein-labeled goat-anti mouse IgG was used to visualize the antigens. For staining F actin cells or tissue sections were incubated for 30 min with rhodamine-phalloidin. Cells or sections were then washed three times in PBS and mounted with 50% glycerol. Slides were viewed with a Zeiss Axiophot, equipped with epifluorescence, photographed with Kodak Tri-X film at an ASA of 1,600, and developed in Accufine (Accufine, Inc., Chicago, IL).

Tissue Sections

150-g rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were perfused by cardiac puncture with 2% formaldehyde in PBS. Tissues were sliced into 1-mm pieces, that were fixed in 2% formaldehyde, PBS for 1 h. The samples were processed through increasing concentrations of sucrose of 15 and 30%, frozen in liquid nitrogen, and 4–6-μm frozen sections were prepared. Sections were incubated with 2% BSA, PBS for 15 min, and further incubated with antibodies as described above.

Immunoblotting Studies

Rat erythrocyte ghosts were prepared by hypotonic lysis (1). Before lysates of MDCK cells and keratinocytes were obtained, cells were first incubated with PBS, 5 mM EDTA, 5 mM diisopropylfluorophosphate for 15 min. Rat brain and rat intestine was removed from a rat perfused with 0.15 M NaCl, 5 mM sodium phosphate, 2.5 mM NaEDTA, 5 mM diisopropylfluorophosphate, pH 7.5. Tissues and cells grown to confluence in 35-mm culture wells were directly dissolved in sample buffer and heated for 10 min. Lysates were forced 10× through a 26-gauge needle to shear DNA. Samples were analyzed by SDS gel electrophoresis with buffers of Fairbanks et al. (11) on 1.5-mm-thick 3.5–17.0% exponential gradient slab gels in 0.2% (wt/vol) SDS. Gels were either stained with Coomassie blue or the separated proteins were electrophoretically transferred to nitrocellulose filters and incubated with affinity-purified antibodies at 0.2–0.5 μg/ml. The nitrocellulose was incubated with anti-brain spectrin IgG or anti-brain adducin IgG as described (6).

In Vivo Phosphorylation

Confluent monolayers were used for phosphorylation studies. MDCK cells were first depleted of phosphate by two 10-min incubations in 130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.6 mM $CaCl_2$, 5 mM dextrose, 30 mM Hepes, pH 7.4. Keratinocytes were cultured for 3 d according to the protocol mentioned above. Cell contact was induced with addition of Ca^{++} to a final concentration of 0.3 mM for 3 h. Cells were then depleted of phosphate by two 10-min incubation in the same medium but without phosphate. From this stage the same protocol was used for MDCK cells and keratinocytes. Labeling was performed by supplementing the same buffer with 200 μCi/ml of [^{32}P]orthophosphate (10 mCi/ml; New England Nuclear, Boston, MA) and incubation for 4 h to equilibrate the intracellular ATP pool. Cells were then washed three times with 150 mM NaCl, 10 mM $NaPO_4$, and 5 mM diisopropylfluorophosphate. Cells were lysed by adding warm lysis buffer and samples were boiled for 5 min. Lysates were sheared, incubated for 1 h with 10 mM of iodoacetamide, and diluted with 4 vol of 150 mM NaCl, 10 mM sodium phosphate, 1 mM NaEDTA, pH 7.4, with additions of 10 mg/ml BSA, 1% Triton X-100, 10 μg/ml leupeptin. Immunoprecipitation was performed by first preabsorbing with rabbit nonimmune IgG for 3 h at 4°C. Protein A–Sepharose was added for 90 min and beads were pelleted by centrifugation. The supernatants were then incubated 14–16 h at 4°C with nonimmune antibody or anti-brain adducin antibody, respectively. Protein A beads were added for 90 min; the beads were washed in 150 mM NaCl, 10 mM sodium phosphate, 0.1% Triton X-100, 1 mM Na EDTA, 1 mM NaN_3, and transferred to fresh tubes. Additional washes were performed with 2 M urea, 1% Triton X-100, 0.1 M glycine, pH 7.0 followed by 0.5 M NaCl, 0.1% Triton X-100, 0.1 mM NaEDTA, 1 mM NaN_3, 10 mM sodium phosphate, pH 7.4 and a final wash with the above buffer without Triton X-100. Pellets were then solubilized in 30 μl of PAGE sample buffer containing 5% SDS, and heated at 70°C for 5 min. Samples were analyzed by electrophoresis and labeled polypeptides visualized by autoradiography.

Results

Characterization of Antibodies against Adducin

Affinity-purified antibodies against brain adducin cross reacted with two polypeptides in red cell ghosts of 103,000 and 97,000 M_r representing the alpha and beta subunits of erythroid adducin. Cross reactivity with both alpha and beta subunits could also be detected in rat duodenum epithelium and MDCK cells. In human epidermal keratinocytes the antibody appears to react only with a single band comigrating with the alpha subunit of erythroid adducin. It is not clear at this point if keratinocytes have a single adducin subunit or if the subunits of human keratinocytes comigrate on these SDS gels. Another possibility is that keratinocytes have an adducin subunit that is not recognized by this particular antibody. The same results have been obtained with both ker-

atinocytes and MDCK cells in terms of localization of adducin. Thus a difference in subunit composition of keratinocyte adducin would have no direct bearing on the conclusions of this study. In brain the antibody has been previously shown to cross react with two subunits representing polypeptides with 109,000/107,000 and 104,000 M_r (2). Antibodies to brain spectrin reacted strongly with a 260,000-M_r polypeptide in brain, duodenum, human epidermal keratinocytes, and MDCK cells (Fig. 1).

Localization of Adducin, Spectrin, and Actin in Rat Tissues

As a first step in understanding possible functions of adducin it was important to determine the distribution of this protein in tissues and evaluate the possibility of colocalization of adducin with spectrin and actin. Adducin is associated with the plasma membrane in those tissues where it is expressed, including epithelial cells of the small intestine, lens cells, and axons of neurons (Fig. 2). In cross sections through peripheral nerve staining of adducin appears in ring-like structures while on longitudinal sections a parallel pattern of stain is observed. A strikingly similar pattern of staining is observed with brain spectrin (Fig. 2). Actin, visualized with rhodamine-phalloidin, also is associated with the axonal membrane (Fig. 2). Actin (9) and spectrin (33, 20) have been previously localized in a continuous pattern similar to that of adducin underlying the axonal plasma membrane.

Adducin in intestinal epithelial cells is limited to the lateral cell borders (Fig. 2). Spectrin and actin are colocalized with adducin in lateral cell borders, but also are present other regions where adducin is absent. Spectrin is present in a uniform distribution along all aspects of the plasma membrane. Actin also is present along the plasma membrane but in addition is intensely labeled at the terminal web (Fig. 2). Similar results for the distribution of spectrin and actin have been obtained by other authors (8, 10).

Adducin is essentially absent from squamous epithelium in rat tongue, even though these cells contain spectrin and actin (Fig. 2). Adducin presumably is lost at some stage during differentiation of keratinocytes since adducin is expressed in cultured human keratinocytes obtained from cell culture that are competent to differentiate in culture (see below). Alternatively, a form of adducin may be expressed in differentiated keratinocytes that is not reactive with this antibody.

Thus adducin and spectrin are both restricted to the plasma membrane and are colocalized along axonal plasma membranes. In intestinal epithelia, adducin exhibits a polarized distribution confined to the lateral cell borders while spectrin is generally distributed on lateral, basal, and apical cell surfaces. Finally in some tissues such as squamous epithelia (Fig. 2) and liver (not shown) spectrin is present in the absence of adducin. These morphological results indicate that spectrin can be expressed in the absence of adducin but adducin was never observed in the absence of spectrin.

Calcium-dependent Localization of Adducin at Sites of Cell–Cell Contact in Cultured Epithelial Cells

The localization of adducin to lateral cell borders of intestinal epithelial cells suggested the possibility that adducin may be preferentially localized at sites of cell–cell contact. To further investigate this hypothesis, localization of adducin was examined in cultured human epidermal keratinocytes (Fig. 3) and MDCK cells (see Fig. 9). Keratinocytes grown in medium with 0.1 mM Ca^{++} have refractile cell borders by light microscopy, indicating that tightly opposed cell junctions have not yet formed (Fig. 4 *b*). Increasing the Ca^{++} concentration to 0.3 mM induces the cells to associate more closely and establish cell–cell contacts (Fig. 4 *d*). Cells grown in 0.03–0.1 mM Ca^{++} for 3 d revealed only a diffuse staining of adducin (Fig. 4). In some cells a punctate pattern of staining was observed concentrated around the nuclei. A

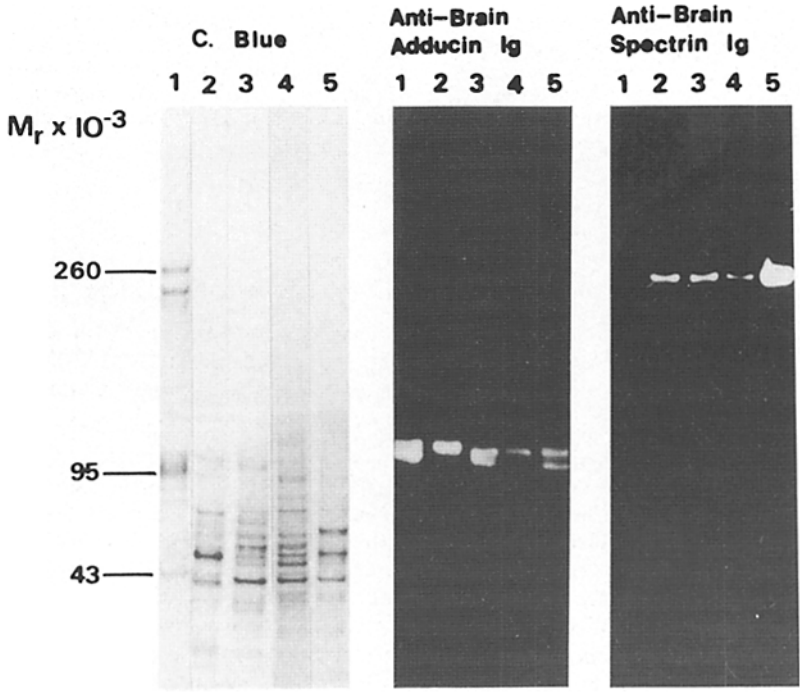


Figure 1. Immunoblot analysis with antibodies to bovine brain adducin or to brain spectrin of human erythrocyte ghosts (lanes *1*), rat brain (lanes *2*), rat intestinal epithelium (lanes *3*), human epidermal keratinocytes (lanes *4*), and MDCK cells (lanes *5*). Samples in the left section were stained with Coomassie blue. Antibodies were visualized with ^{125}I–protein A. (For details refer to Materials and Methods.)

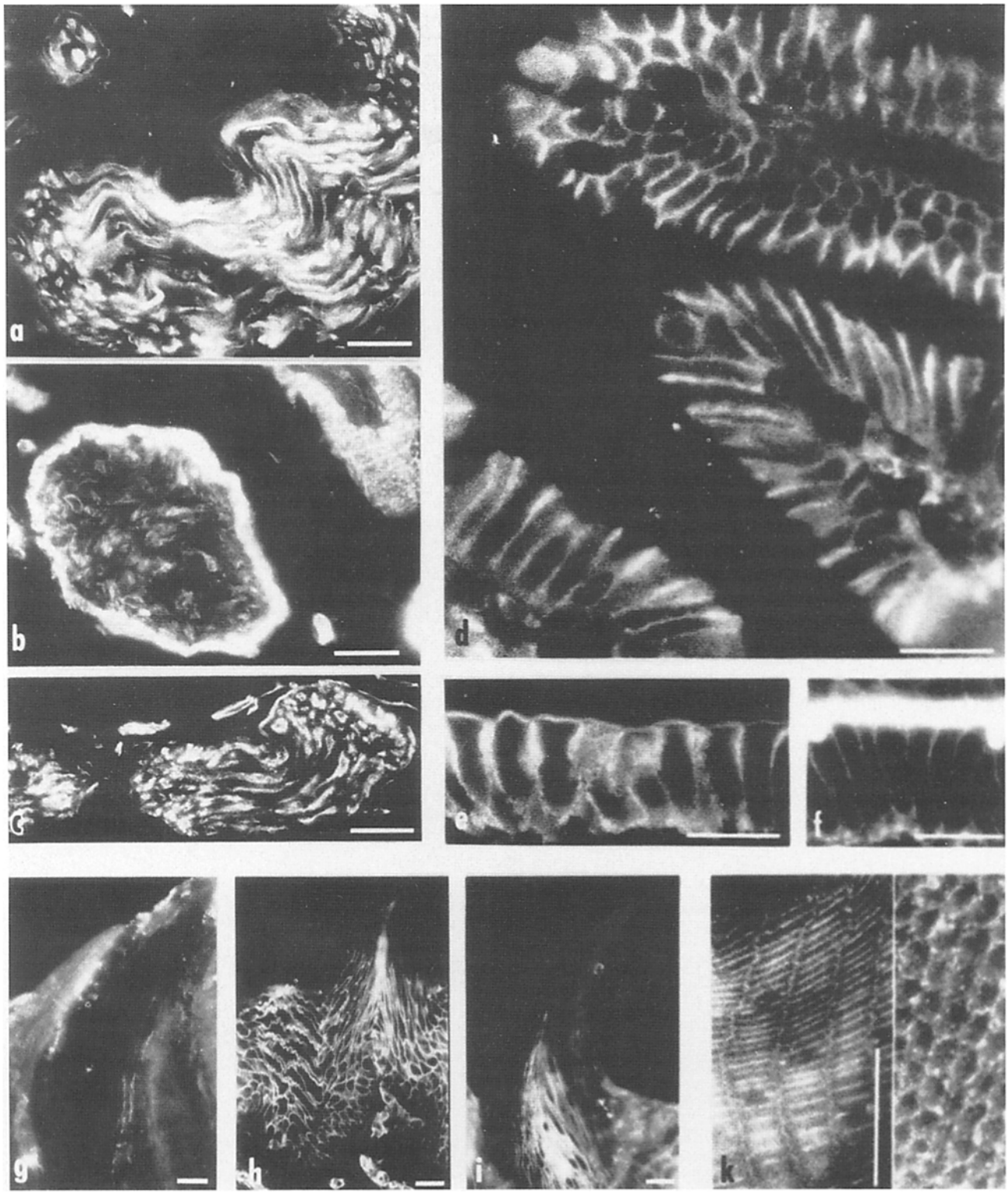


Figure 2. Localization of adducin (*a*, *d*, *g* and *k*), actin (*b*, *f*, and *h*), and spectrin (*c*, *e*, and *i*) by fluorescent microscopy using affinity-purified antibodies (adducin, spectrin) or rhodamine phalloidin (actin) in cryostat sections of rat lingual nerve (*a*, *b*, and *c*), rat duodenum (*d*, *e*, and *f*), filiform papillae of rat tongue (*g*, *h*, and *i*), and rat lens (*k*). Adducin, spectrin, and actin were colocalized in neurites of the peripheral nerve in an area near the plasmalemma of the nerve fibers. In duodenum adducin was confined to the lateral cell borders while actin and spectrin were present along the apical and basolateral membrane. Adducin was virtually absent in adult filiform papillae, while spectrin and actin could be detected. In rat lens adducin outlines the cell borders of lens cells. Bars, 20 μm.

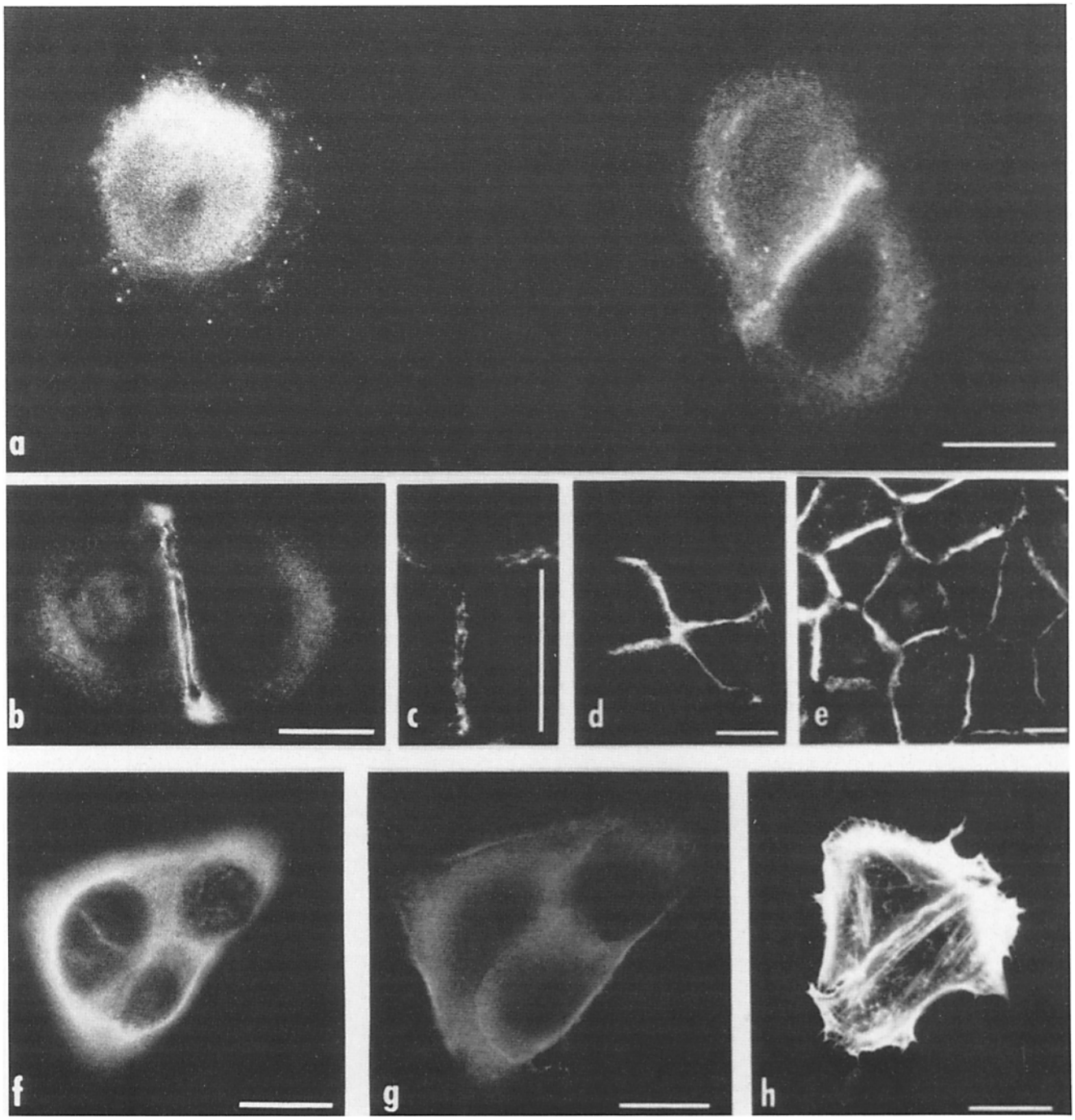


Figure 3. Fluorescent microscopy of human epidermal keratinocytes grown in 0.3 mM Ca^{++} with Ig against brain adducin (*a–e*), brain-spectrin (*f* and *g*), and rhodamine-phalloidin (*h*). Adducin showed a diffuse fluorescence in single cells but as cells attach, adducin was concentrated at sites of cell–cell contact (*a*, *b*, *d*, and *e*). In some areas parallel pairs of adducin containing structures were visible (*c*). In contrast to adducin, spectrin was localized throughout the cell near the cell membrane as was demonstrated by focusing through different levels (*f* and *g*). Actin (*h*) was present in a diffuse distribution as well as in centrally located stress fibers. (See Materials and Methods for further details.) Bars, 20 μm.

marked change of the adducin distribution occurs upon elevation of Ca^{++} to 0.3 mM. Cells which were attached to other cells exhibited a concentration of adducin at sites of cell–cell contact while cells whose cell borders were not in contact with other cells continued to show a diffuse distribution (Fig. 3 *a*). In some areas adducin staining was confined to punctate structures at sites of cell–cell contact (Fig. 3 *c*). The localization of adducin at sites of cell–cell contact could be observed at different stages in cell colonies. As soon as two cells appeared to be in contact, adducin was present in high concentration at the contact sites, (Fig. 3, *a* and *b*) and could also be detected at such sites in multiple cell colonies (Fig. 3 *d*) up to confluent monolayers (Fig. 3 *e*).

Several findings support the conclusion that the concentra-

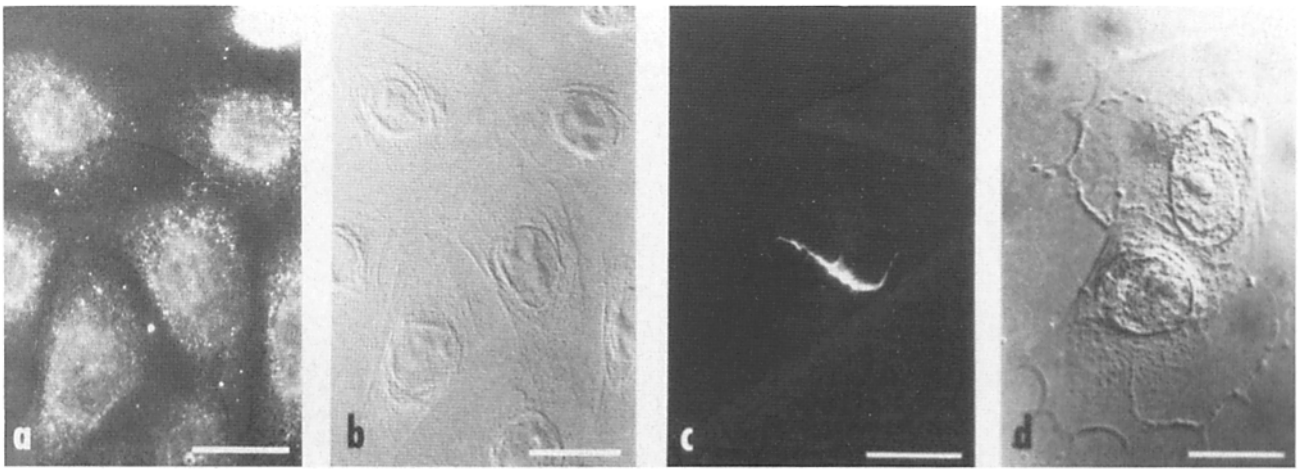


Figure 4. Effect of different Ca^{++} levels on the distribution of adducin in human epidermal keratinocytes. Cells were grown either in 0.1 mM Ca^{++} (*a* and *b*) or in 0.3 mM Ca^{++} (*c* and *d*). In 0.1 mM Ca^{++} concentration (*a* and *b*) cells had refractile cell borders and were growing in loose colonial morphology shown by differential interference contrast microscopy (*b*). Antibodies against adducin in these cells showed a diffuse fluorescence (*a*). In 0.3 mM calcium, cells were associated closely (*d*) and adducin was present in high concentrations at sites of cell-cell-contact (*c*). (For details see Materials and Methods.) Bars, 20 μm.

tion of adducin is increased at sites of cell-cell contact and that the apparent increase in fluorescence is not due to the sum of the staining from two membranes. (*a*) Antibodies to spectrin which stain the entire cytoplasmic surface of the cell membrane, do not show any concentration of spectrin at sites of cell-cell contact. The fluorescence is evenly distributed as can be estimated by focusing through different planes of the cells (Fig. 3, *f* and *g*). (*b*) With preimmune serum no stain at sites of cell-cell contact was detected (data not shown). (*c*) In some cell colonies an intercellular gap was present showing intensive staining at both membranes opposing each other (Fig. 3, *b* and *c*). The occurrence of an intercellular space may be due to fortuitous viewing between opposed cells along an axis perpendicular to the plane of cells.

Actin was detected in keratinocytes with rhodamine-labeled phalloidin, which is a specific probe for F actin (42). In keratinocytes cultured at 0.3 mM Ca^{++} actin was diffusely distributed in the cortical cytoplasm and in addition centrally located in stress fibers. Adducin was not detected in association with actin stress fibers (Fig. 3 *h*). Spectrin also was visualized in keratinocytes (Fig. 3, *f* and *g*), where it is uniformly distributed along the plasma membrane. Spectrin also was observed apparently in the cytoplasm of these cells (Fig. 3, *f* and *g*). However, the impression from viewing cells at different levels of focus is that the staining with spectrin was limited to the plasma membrane. The apparent cytoplasmic staining in Fig. 3 is most likely due to superimposed membranes. Fig. 2 represents frozen sections of tissues, while Fig. 3 is produced with intact tissue culture cells. The differences between Fig. 2, where spectrin is limited to plasma membranes, and Fig. 3 thus is most likely the consequence of improved resolution of membrane surfaces in sections. The results with cultured cells indicate that spectrin can be expressed in the absence of adducin but adducin was never observed in the absence of spectrin. The same result was observed in sections of tissues (Fig. 2).

Distribution of Adducin, Vinculin, and Desmoplakin at Different Times after Elevation of Ca^{++} and at Different Levels of Ca^{++}

Formation of vinculin and desmoplakin-containing junctions can be induced in cultured keratinocytes by shifting the extracellular Ca^{++} concentration from low to high levels (16, 17, 30, 39). We wanted to compare the occurrence of these junctions to the appearance of adducin at sites of cell-cell contact after elevation of the Ca^{++} concentration.

Cells were cultured in low Ca^{++} medium for 3 d and shifted to a higher Ca^{++} concentration for various times. After fixation and permeabilization cells were double-stained either for adducin and vinculin or for adducin and desmoplakin (Fig. 5). Adducin was present at sites of cell-cell contact within 15 min after induction in most cells. The process of adducin concentration at sites of cell-cell contact was completed between 15 and 45 min after addition of calcium, depending on the proximity of cell borders.

Double labeling for vinculin and adducin showed that intercellular junctions containing vinculin formed in a few cells within 15 min and were colocalized with adducin at sites of cell-cell contact. However, adducin was already present at sites of cell-cell contact in cells where vinculin-containing junctions were not found at this early time point (Fig. 5, *e* and *f*). 1 h after elevation of calcium, vinculin and adducin were colocalized at sites of cell-cell contact in all cells (Fig. 5, *i* and *k*). A similar result was obtained for keratinocytes double-labeled with antibodies against adducin and desmoplakin. Adducin was present at most sites of cell-cell contact within 15 min but staining for desmoplakin revealed the presence of only a few desmosomes at these sites (Fig. 5, *g* and *h*). The formation of desmosomes at all sites of cell-cell contact required the presence of 1.1 mM Ca^{++} for 2 h (Fig. 5, *l* and *m*). At this time adducin and desmoplakin were colocalized at sites of cell-cell contact.

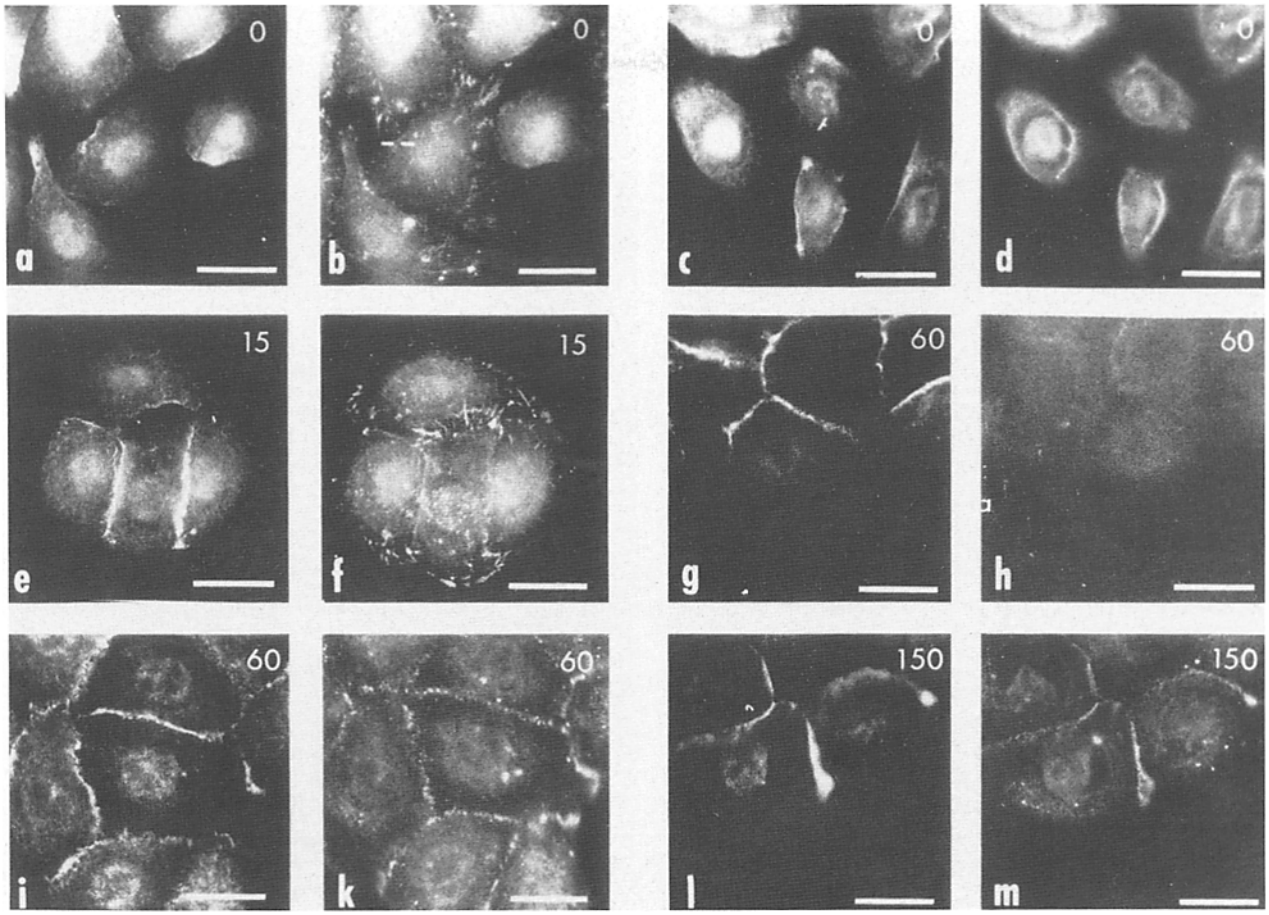


Figure 5. Double immunofluorescence of epidermal keratinocytes stained for adducin (*a*, *e*, and *i*) and vinculin (*b*, *f*, and *k*), or adducin (*c*, *g* and *l*) and desmoplakin (*d*, *h*, and *m*) at various time points after elevation of Ca^{++}. Cells were cultured for 3 d at 0.1 mM Ca^{++}. Ca^{++} then was raised to 1.1 mM for times (indicated in minutes in right upper corner). Adducin was present at cell–cell contact sites before vinculin or desmoplakin-containing junctions were formed. Bar, 20 μm.

Generally adducin is present at intercellular sites before formation of junctions containing vinculin and desmoplakin.

The difference in rates redistribution of adducin and other junctional proteins was quantitative and not absolute. Thus it is conceivable that vinculin or desmoplakin may be required for initial attachment of a small population of adducin at sites of cell–cell contact. Such a possibility is ruled out by comparison of the effect of calcium concentration on redistribution of adducin, vinculin, and desmoplakin to cell junctions. A marked difference was noted for the staining of these proteins at medium (0.3 mM) and high (1.1 mM) concentrations of calcium (Fig. 6). At 0.3 mM Ca^{++} adducin was shifted to sites of cell–cell contact in all adherent cells while staining for vinculin and desmoplakin remained the same as in low calcium. Vinculin was perinuclear and associated with attachment plaques at the central aspect of cells, while desmoplakin exhibited a diffuse pattern with a concentration in the perinuclear region. Incubation with 0.3 mM Ca^{++} for 3 h did not induce formation of vinculin and desmoplakin containing junctions. Only adducin could be detected in high concentrations at sites of cell–cell contact in these cells (Fig. 6, *a–d*). Vinculin and desmoplakin were not observed at sites of cell–cell contact even after a 24-h incubation of cells in 0.3 mM Ca^{++} (not shown). A different keratinocyte strain required a lower Ca^{++} concentration for formation of adjacent cell borders. In this strain close cell–cell contact was present at 0.1 mM Ca^{++}. Cells were maintained for several weeks and passaged at least five times at this Ca^{++} concentration. In this cell line adducin was present in high concentrations at sites of cell–cell contact at low Ca^{++} concentration while staining for vinculin and desmoplakin could not be detected at intercellular sites (data not shown). Vinculin- and desmoplakin-containing junctions were induced within 3 h by addition of Ca^{++} to a final concentration of 1.1 mM in both cell lines. Thus the concentration of adducin at sites of cell–cell contact consistently required lower concentrations of Ca^{++} than the formation of specialized intercellular junctions (Fig. 6, *e–h*).

The concentration of adducin at sites of cell–cell contact after addition of Ca^{++} is most likely due to redistribution of existing adducin rather than proteolytic processing of adducin or stimulation of adducin synthesis. Adducin appears at contact sites within 15 min, which is an unusually short time for induction of protein synthesis. No increase in steady-state

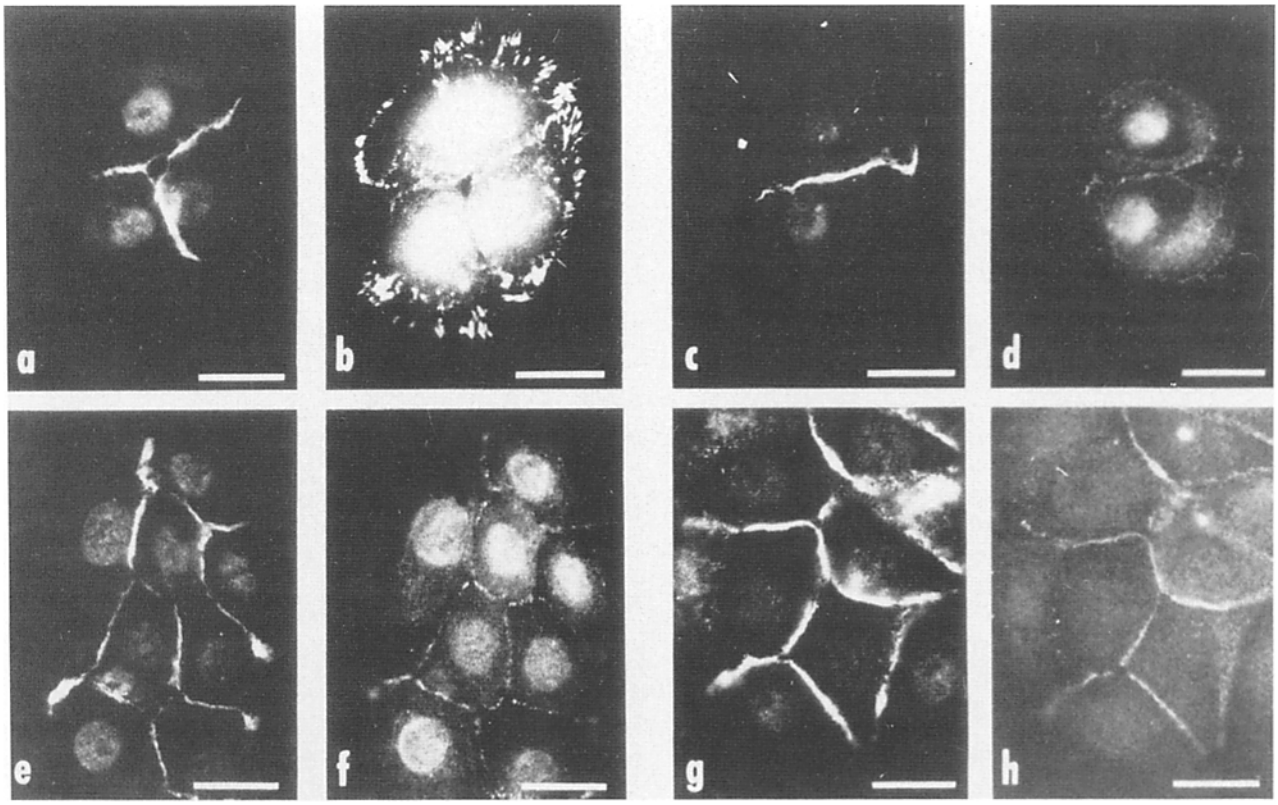
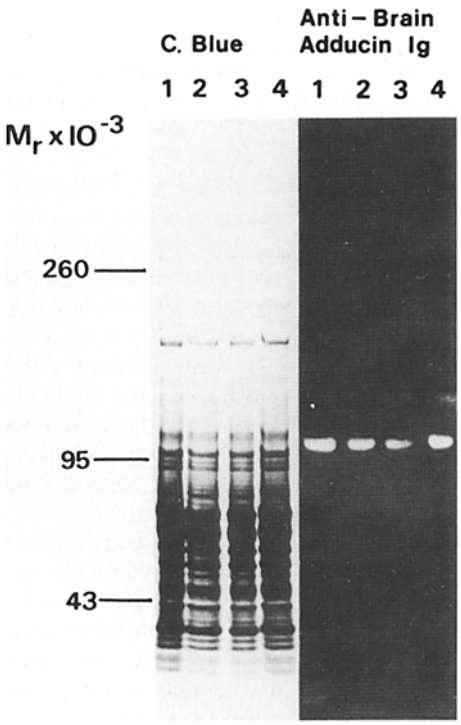


Figure 6. Double-label immunofluorescence of human epidermal keratinocytes with antibodies to adducin (*a* and *e*) and vinculin (*b* and *f*), or adducin (*c* and *g*) and desmoplakin (*d* and *h*) at different Ca^{++} levels. Cells were grown in 0.1 mM Ca^{++} and shifted to 0.3 mM Ca^{++} (*a–d*) or 1.1 mM Ca^{++} (*e–h*) for 3 h before fixation. Adducin was present at sites of cell–cell contact at 0.3 mM Ca^{++} (*a* and *c*). Note that antibodies to vinculin stained attachment plaques but vinculin or desmoplakin-containing junctions were not formed in 0.3 mM Ca^{++}. Shifting the Ca^{++} level to 1.1 mM for 3 h produced adducin staining at cell–cell contact sites and vinculin and desmoplakin-containing junctions in all contiguous cells. Bar, 20 μm.



levels of adducin or decrease in the relative molecular mass of the adducin polypeptides could be detected by immunoblots of adducin in keratinocytes at 0.03, 0.1, 0.3, and 1.1 mM Ca^{++} concentrations (Fig. 7). Moreover, adducin still appeared at sites of cell–cell contact when keratinocytes were incubated for 30 min in 0.3 mM Ca^{++} with puromycin at concentrations (20 μg/ml) that inhibited >95% of protein synthesis (data not shown). These results do not address the possibility that calcium may modify the relative rates of degradation or synthesis of adducin. Answering these questions will require metabolic labeling of cells and pulse–chase experiments, and are beyond the scope of this report.

Figure 7. Immunoblot analysis of adducin in keratinocytes after culture at various Ca^{++} concentrations. Keratinocytes grown at various Ca^{++} levels were compared by immunoblots using antibody against brain adducin. Keratinocytes were cultured for 3 d in 35-mm culture wells in 0.03 mM Ca^{++} (lane *1*) or 0.1 mM Ca^{++} (lanes *2–4*), and then raised to 0.3 mM Ca^{++} (lane *3*) and 1.1 mM Ca^{++} (lane *4*) for 3 h. Cells were preincubated in PBS with DFP for 20 min (with the Ca^{++} concentration maintained) and then directly lysed into SDS-PAGE buffer and analyzed by SDS gel PAGE. Gels were either stained with Coomassie blue or polypeptides were transferred to nitrocellulose and incubated with antibody against brain adducin.

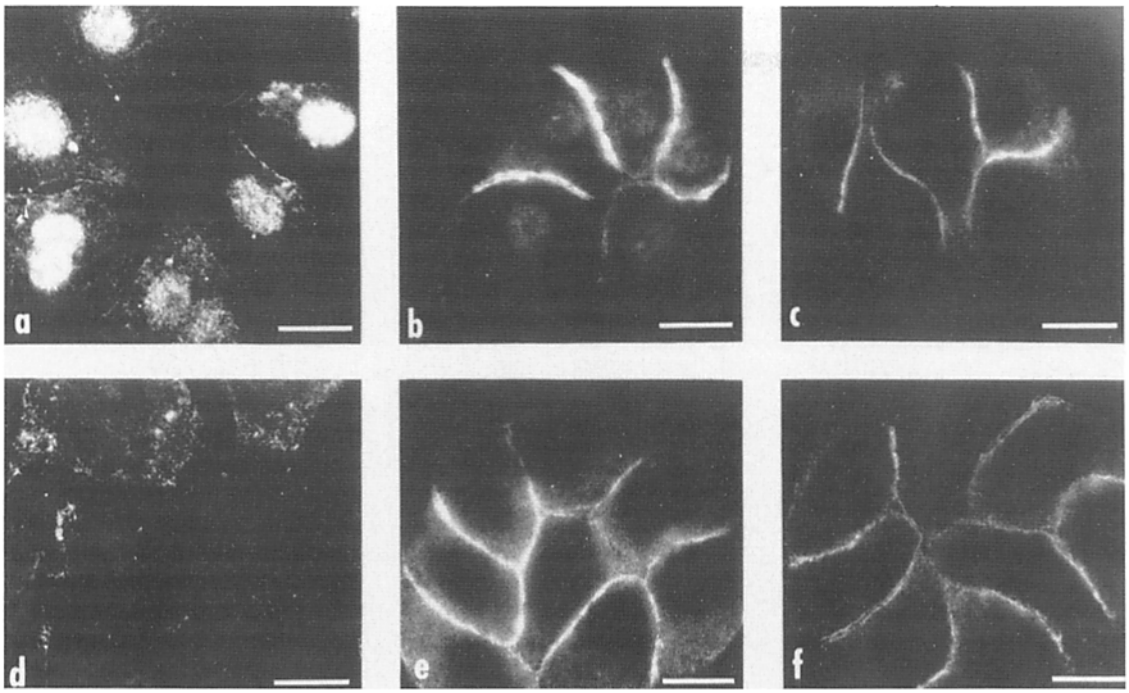


Figure 8. Adducin and spectrin were colocalized in a detergent-resistant state at cell–cell attachment sites. Keratinocytes were cultured for 3 d in 0.1 mM Ca^{++}, followed by culture for 3 h with Ca^{++} concentrations of either 0.1 mM (*a* and *d*), 0.3 mM (*b* and *e*), or 1.1 mM (*c* and *f*). Keratinocytes were extracted at 24°C with 0.25% Triton X-100 for 5 min before fixation with 2% formaldehyde and staining with Ig against adducin (*a*, *b*, and *c*) and spectrin (*d*, *e*, and *f*). In 0.1 mM Ca^{++} (*a* and *d*) staining for adducin and spectrin was reduced. At Ca^{++} levels of 0.3 mM (*b* and *e*) and 1.1 mM (*c* and *f*), spectrin was less extractable from the membrane, but only at sites of cell–cell contact, where spectrin was co-localized with adducin. Bars, 15 μm.

Spectrin and Adducin Are Colocalized in a Detergent-insoluble Form at Cell–Cell Contact Sites

Spectrin has been observed to exhibit increased resistance to extraction in MDCK cells after formation of cell–cell contact (25, 26). Reduced extraction of spectrin at cell junctions may reflect formation of a stable spectrin-actin lattice analogous to the structure of erythrocyte membrane skeletons. Adducin in erythrocytes is tightly associated with the membrane skeleton and is not solubilized by Triton X-100 at physiological concentrations of ions (12). We wanted to determine if adducin at sites of cell–cell contact also was inextractable in detergent and if conversion of spectrin to a detergent-insoluble form occurred in parallel with redistribution of adducin to sites of cell–cell contact. Therefore keratinocytes were cultured at low (0.1 mM), medium (0.3 mM), and high (1.1 mM) Ca^{++} levels and extracted with Triton X-100 before fixation. At low levels of Ca^{++} when cells are not in contact the staining for spectrin and adducin was diffuse and reduced by extraction with Triton X-100 (Fig. 8, *a* and *d*). Addition of Ca^{++} to a final concentration of 0.3 mM (Fig. 8, *b* and *e*) or 1.1 mM (Fig. 8, *c* and *f*) resulted in retention of spectrin and adducin at areas of cell–cell contact but extraction of spectrin in areas that had no contact with other cells. Staining of spectrin in detergent-extracted cells grown in medium and high calcium showed a striking similarity to staining of adducin. Adducin and spectrin were both present in a discontinuous layer of small plaques in the area beneath the membrane with counterparts in the neighboring cells.

The solubility of spectrin in detergent exhibited no difference in medium or high Ca^{++} concentrations. Transformation of spectrin to a detergent-insoluble form was independent of the presence of adherens junctions or desmosomes since vinculin and desmoplakin were not present at sites of cell–cell contact at medium Ca^{++} concentrations (Fig. 6). It will be important in future experiments to determine the relative stoichiometries of adducin and spectrin at cell–cell contact sites and to visualize these proteins at an ultrastructural level.

Redistribution of Adducin from Sites of Cell–Cell Contact by Phorbol Ester

Adducin is a substrate for protein kinase C in vitro and in vivo (2, 5, 21, 32) although functional consequences of phosphorylation of adducin are not yet known. It was of interest to determine if stimulation of protein kinase C by the tumor-promoting phorbol ester (TPA) would have any effect on the localization of adducin at sites of cell–cell contact. Keratinocytes cultured for 3 d in 0.1 mM Ca^{++} were induced to form cell–cell contacts by elevation of the calcium concentration. After induction of cell–cell contact cells were incubated for various times with 26 nM TPA, which activates protein kinase C (28). Observation of cells by phase contrast after stimulation with TPA revealed only subtle changes in cell morphology. With time lapse photography an increase in ruffling could be detected at sites of cell–cell contact after 4 h. However, keratinocytes failed to form a "bizarre" morphology with rounding of cells and formation of protrusions as seen in MDCK cells and other cell lines (35).

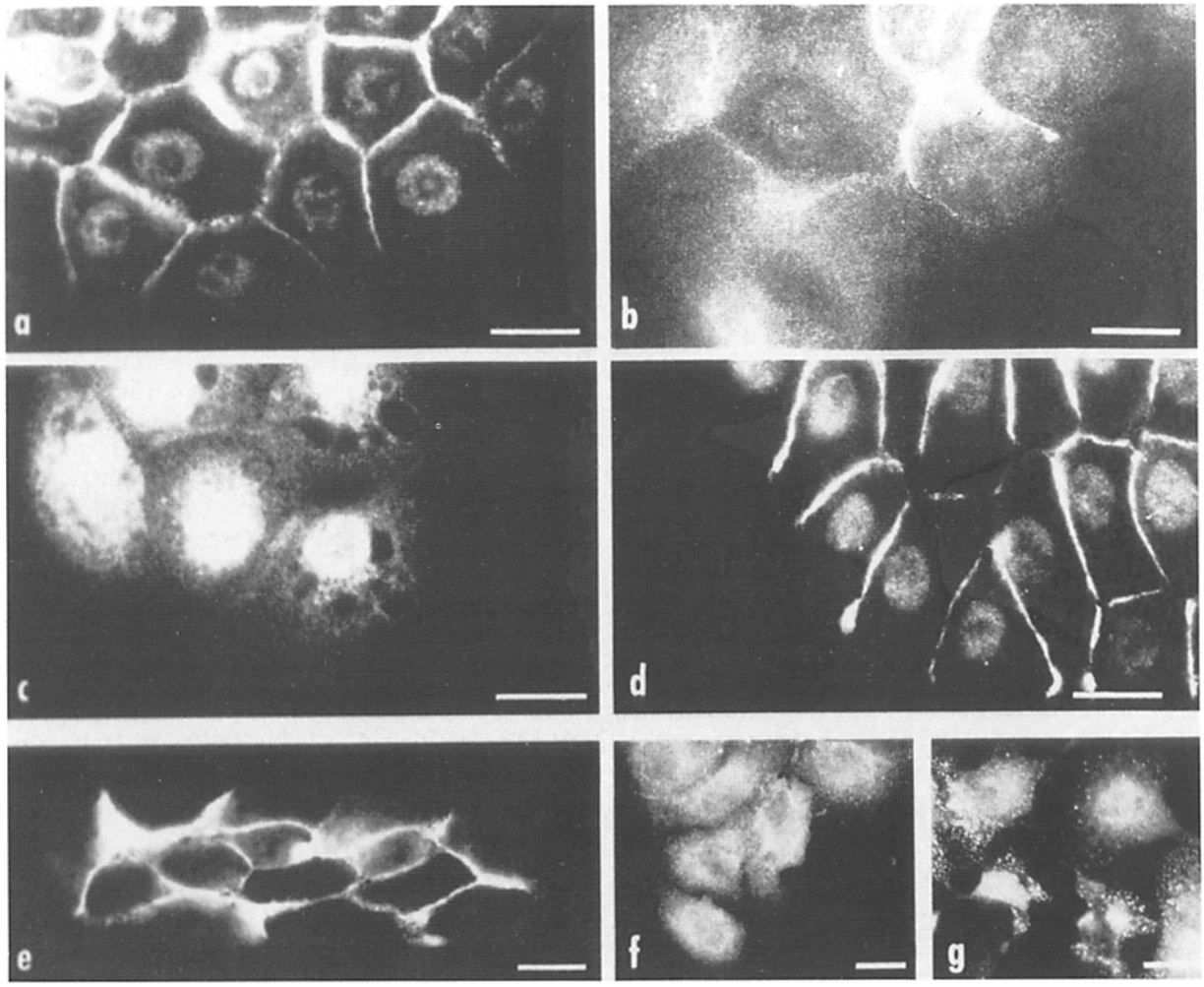


Figure 9. Tumor-promoting phorbol ester (TPA) induced redistribution of adducin from sites of cell–cell contact. Immunofluorescence of human epidermal keratinocytes (*a–d*) and MDCK cells (*e–g*) treated with 26 nM phorbol ester dissolved in DMSO (final concentration 0.025%) for different time points. Redistribution of adducin away from sites of cell–cell contact required several hours. 30 min after stimulation (*a*) no obvious changes in adducin localization were apparent compared to control (10 h 0.025% DMSO) (*d*). Partial redistribution of adducin occurred after 2 h (*b*) and complete redistribution with diffuse staining for adducin occurred after 10 h (*c*). In MDCK cells, depletion of adducin from sites of cell–cell contact appeared faster. 4 h after stimulation complete redistribution with diffuse staining (*f*) and gross morphological changes (*g*) were present as compared to control (4 h DMSO 0.025%) (*e*). Controls shown in *d* and *e* are representative; no change was noted for the localization of adducin after incubation with 0.025% DMSO for different times (between 0.5 and 10 h). Bars, 20 μm.

Keratinocytes exhibited a time-dependent redistribution of adducin after addition of TPA (Fig. 9, *a–c*). After 30 min of phorbol stimulation adducin remained at sites of cell–cell contact but showed a slight increase in cytoplasmic fluorescence in comparison to control cells incubated with equal amounts of DMSO (Fig. 9 *d*), the solvent which was used to dissolve phorbol ester. 2 h after addition of phorbol ester, adducin was still present at some areas of cell–cell contact, although in a reduced concentration. 10 h with phorbol ester were required for complete redistribution of adducin away from the cell membrane sites into a diffuse intracellular location. Immunoblots showed that steady-state levels of adducin were similar in stimulated and unstimulated cells and that adducin was not proteolysed to lower relative molecular mass polypeptides during exposure of cells to TPA (data not shown).

The effects of TPA on adducin distribution were also examined in MDCK cells. In MDCK cells exposed to TPA, adducin was redistributed away from sites of cell–cell contact before gross morphological changes occurred (Fig. 9, *f* and *g*).

To determine if phosphorylation of adducin occurred in TPA-treated cells, MDCK cells and keratinocytes were metabolically labeled with [^{32}P]orthophosphate followed by treatment with or without phorbol ester. Treatment of MDCK cells and human epidermal keratinocytes with 26 nM phorbol ester results in phosphorylation of polypeptides of the same relative molecular mass as adducin that were immunoprecipitated from cell lysates with antibodies against brain adducin (Fig. 10). Adducin phosphorylation in keratinocytes and MDCK cells was rapid with maximal phosphorylation within 5 min after addition of phorbol ester. Cells were also incubated with DMSO to assure that phosphorylation was induced only by phorbol ester. DMSO had no effect on phos-

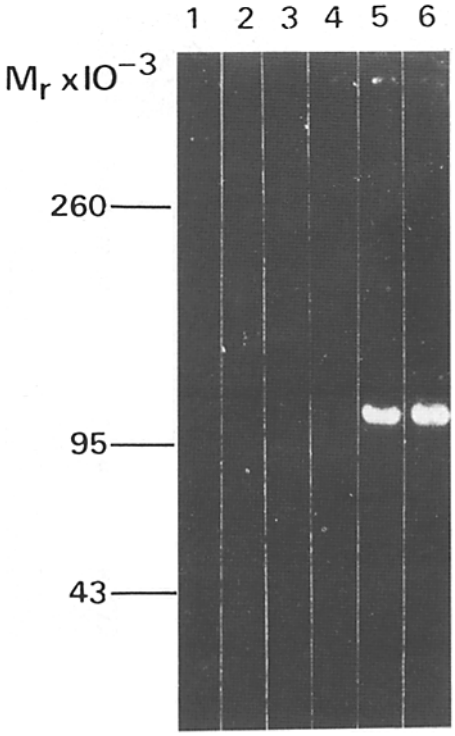


Figure 10. Tumor promoting phorbol ester (TPA) stimulated phosphorylation of adducin in living cells. Immunoprecipitates of keratinocyte lysates with nonimmune serum (lanes *1–3*) and anti-brain adducin antibodies (lanes *4–6*) were analyzed by SDS electrophoresis and autoradiography. Cells were treated with DMSO (final concentration of 0.025%) for 30 min (lanes *1* and *4*) or with 26 nM phorbol ester dissolved in DMSO (final concentration of 0.025%) added for 5 min (lanes *2* and *5*) and 30 min (lanes *3* and *6*). Adducin was an in vivo substrate for protein kinase C, and was almost fully phosphorylated within the first 5 min.

phorylation (Fig. 10). The phorbol ester–dependent phosphorylation of adducin in intact cells is consistent with the hypothesis that phosphorylation is the signal responsible for redistribution of adducin from sites of cell–cell contact. Definitive proof that phosphorylation of adducin is the only step required for redistribution will require additional experiments.

Discussion

This report describes the localization of adducin to plasma membranes of a variety of tissues and the discovery that adducin is concentrated at sites of cell–cell contact in the epithelial tissues where it is expressed. The presence of adducin at cell–cell contact sites of cultured epithelial cells requires extracellular Ca^{++}, occurs independently of formation of desmosomal and adherens junctions, and is reversed by treatment of cells with phorbol ester. Adducin in tissues and cultured cells always was observed in association with spectrin and actin, although spectrin and actin also occur in the absence of adducin. In sections of intestinal epithelial cells spectrin was evident on all plasma membrane surfaces while adducin was restricted to the lateral cell borders. Adducin also was not detected in association with actin stress fibers in cultured cells. These findings are in agreement with the results of in vitro assays indicating that adducin associates with spectrin–actin complexes but only weakly with actin alone (2, 13, 14).

Tumor promotors have dramatic effects on cells in tissue culture, presumably due to activation of the protein kinase C pathway. Various intracellular proteins such as vinculin, actin, and alpha-actinin have been shown to be redistributed after phorbol stimulation (23, 35). Vinculin is a substrate for protein kinase C (40) although effects of phosphorylation on vinculin activity are not known. Adducin is an additional target for protein kinase C and is a candidate to mediate some of the effects of phorbol esters on cell morphology. Adducin is a substrate in vitro and in vivo for protein kinase C (2, 5, 21, 32) and was demonstrated in this study to be phosphorylated in cells and redistributed from sites of cell–cell contact by treatment with TPA. Redistribution of adducin was relatively slow compared to phosphorylation. It is not known at this point if the slow response to phosphorylation is due to involvement of additional proteins, or if this reflects the rate of turn over of adducin at the contact sites. The simplest explanation for the effects of phorbol ester would be that phosphorylation of adducin by protein kinase C lowers the affinity of adducin for the putative membrane attachment site at cell–cell contact sites (see below). Phosphorylation thus would cause adducin either to dissociate and/or not associate with contact sites. This hypothesis may be evaluated by microinjection into cells of labeled phospho- and dephospho-adducin and determining where these forms of adducin distribute.

Spectrin and adducin are both present in a detergent-insoluble form at cell–cell contact sites. A stable organization of spectrin at cell junctions has been noted previously (25, 26). Adducin is a candidate to catalyze formation of an insoluble spectrin lattice after occurrence of cell–cell contact. Biochemical data suggest an organizing function for adducin in promoting association of spectrin and actin (2, 13, 14). Adducin binds with high affinity to spectrin–actin complexes, thus forming a ternary actin–spectrin–adducin complex. In addition adducin can recruit additional spectrins to the spectrin–actin complex. These activities of adducin provide branch-points in spectrin-actin polymers and may be important in assembly of the spectrin-actin network visualized in erythrocytes (4, 22, 36). Adducin, by analogy with the erythrocyte system, could also convert spectrin of other cells into an organized, detergent insoluble network at cell–cell junctions.

The high concentration of adducin at sites of cell–cell contact indicates that a targeting process, perhaps mediated by a protein, is involved in localizing adducin to these specialized membrane domains. Adducin may interact with phosphatidylserine (41), although association with a phospholipid would not be expected to be sufficient for selective targeting to particular regions of the plasma membrane. Another possibility for a targeting mechanism is that adducin interacts selectively with a membrane protein that is concentrated at contact sites. The "receptor" for adducin at cell contact sites may be involved in calcium-dependent cell–cell recognition. Candidate membrane proteins for association with adducin at contact sites would include cell adhesion molecules such as the cadherin family of integral glycoproteins present in areas of cell–cell contact (38).

The distribution of adducin and spectrin indicates a spec-

trin-based intercellular contact distinct from known intercellular junctions that is independent of assembly of desmosomes and adherens junctions. A highly speculative proposal for steps in assembly of the adducin/spectrin adhesion site is: (*a*) An integral membrane protein or family of proteins becomes concentrated at sites of cell-cell contact due to calcium-dependent extracellular interactions; (*b*) Adducin associates with the cytoplasmic domain of the integral membrane protein(s); (*c*) Membrane-associated adducin promotes assembly of a spectrin-actin lattice analogous to the structure visualized in erythrocyte membranes. The ability of adducin to promote assembly of an extended spectrin lattice would be expected to be highly dependent on the local concentration of membrane receptors which in turn would depend on extracellular interactions with adjacent cells. Conversely, the stability of the cell-cell contact would be enhanced by an intracellular mechanism for stabilizing receptor clusters and allowing multipoint attachments between cells. Thus both external cell-cell contacts and intracellular lattice assembly would enhance each other and would be expected to occur in a highly cooperative manner. Assembly of a spectrin-lattice at cell-cell contacts could also increase mechanical stability of the membrane and provide a mechanism for immobilization of other membrane proteins involved in construction of cell junctions. An initial experimental challenge in testing these ideas is to identify the putative membrane receptor for adducin.

Adducin is not expressed in all epithelial tissues, and is in low abundance in differentiated squamous epithelia (Fig. 2), liver, and most cells of the kidney (not shown). Adducin is expressed in rapidly dividing epithelial cells of the intestine and in cultured keratinocytes even though adducin is lacking in more differentiated keratinocytes. It is possible that adducin has an initial role in assembly of cell junctions, but is lost after completion of these structures. In this case, adducin would be analogous to the scaffolding protein of T4 bacteriophage that is involved in construction of the viral heads but is not a component of the final structure.

Technical help of Rick E. Payne and assistance of Jodi Telander in preparation of the manuscript are gratefully acknowledged.

This research was supported in part by grants from National Institutes of Health to V. Bennett (GM33996 and AM19808) and E. O'Keefe (AR 25871 and AR 36497).

References

1. Bennett, V. 1983. Proteins involved in membrane-cytoskeleton association in human erythrocytes: spectrin, ankyrin, and band 3. *Methods Enzymol.* 96:313-324.
2. Bennett, V., K. Gardner, and J. P. Steiner. 1988. Brain adducin: a protein kinase C substrate that may mediate site directed assembly at the spectrin-actin junction. *J. Biol. Chem.* 263:5860-5869.
3. Boyce, S. T., and R. G. Ham. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81(Suppl.): 33s-40s.
4. Byers, T. J., and D. Branton. 1985. Visualization of the protein associations in the erythrocyte membrane skeleton. *Proc. Natl. Acad. Sci. USA.* 82:6153-6157.
5. Cohen, C. M., and S. F. Foley. 1986. Phorbol ester- and Ca^{++}-dependent phosphorylation of human red cell membrane skeletal proteins. *J. Biol. Chem.* 261:7701-7709.
6. Davis, J., and V. Bennett. 1982. Microtubule-associated protein, a microtubule-associated protein from brain, is immunologically related to the a subunit of erythroid spectrin. *J. Biol. Chem.* 257:5816-5820.
7. Davis, J., and V. Bennett. 1983. Brain spectrin. *J. Biol. Chem.* 258:7757-7766.
8. Drenckhahn, D., and V. Bennett. 1987. Polarized distribution of M_r 210,000 and 190,000 analogs of erythrocyte ankyrin along the plasma membrane of transporting epithelia, neurons and photoreceptors. *Eur. J. Cell Biol.* 43:479-486.
9. Drenckhahn, D., and H. W. Kaiser. 1983. Evidence for the concentration of F-actin and myosin in synapses and in the plasmalemmal zone of axons. *Eur. J. Cell Biol.* 31:235-240.
10. Drenckhahn, D., and R. Dermietzel. 1988. Organization of the actin filament cytoskeleton in the intestinal brush border: a quantitative and qualitative immunoelectron microscope study. *J. Cell Biol.* 107:1037-1048.
11. Fairbanks, G., T. Steck, and D. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606-2617.
12. Gardner, K., and V. Bennett. 1986. A new erythrocyte membrane associated protein with calmodulin binding activity. *J. Biol. Chem.* 261: 1339- 1348.
13. Gardner, K., and V. Bennett. 1987. Modulation of spectrin-actin assembly by erythrocyte adducin. *Nature (Lond.).* 328:359-362.
14. Gardner, K., and V. Bennett. 1988. Erythrocyte adducin: a new calmodulin-regulated membrane skeletal protein that modulates spectrin actin assembly. *In* Signal Transduction in Cytoplasmic Organization and Cell Motility. Alan R. Liss, Inc., New York. 293-311.
15. Glenney, J. R., and P. Glenney. 1983. Fodrin is the general spectrin-like protein found in most cells whereas spectrin and the TW protein have a restricted distribution. *Cell.* 34:503-512.
16. Green, K. J., B. Geiger, C. R. Jones, J. C. Talian, and R. D. Goldman. 1987. The relationship between intermediate filaments and microfilaments before and during the formation of desmosomes and adherens-type junctions in mouse epidermal keratinocytes. *J. Cell Biol.* 104:1389-1402.
17. Hennings, H., and K. Holbrook. 1983. Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. *Exp. Cell Res.* 143:127-142.
18. Koob, R., M. Zimmermann, W. Schoner, and D. Drenckhahn. 1987. Colocalization and coprecipitation of ankyrin and Na/K ATPase in kidney epithelial cells. *Eur. J. Cell Biol.* 45:230-237.
19. Deleted in proof.
20. Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631-643.
21. Ling, E., K. Gardner, and V. Bennett. 1986. Protein kinase C phosphorylates a recently identified membrane skeleton-associated calmodulin-binding protein in human erythrocytes. *J. Biol. Chem.* 261:13875-13878.
22. Liu, S., L. Derik, and J. Palek. 1987. Visualization of the hexagonal lattice in the erythrocyte membrane skeleton. *J. Cell Biol.* 104:527-536.
23. Meigs, J. B., and Y. L. Wang. 1986. Reorganization of actin, a-actinin and vinculin induced by a phorbol ester in living cells. *J. Cell Biol.* 102:1430-1438.
24. Mische, S., M. Mooseker, and J. Morrow. 1987. Erythrocyte adducin: a calmodulin-regulated actin-bundling protein that stimulated spectrin-actin binding. *J. Cell Biol.* 105:2827-2849.
25. Nelson, W. J., and P. J. Veshnock. 1986. Dynamics of membrane-skeleton (fodrin) organization during development of polarity in madin-darby canine kidney epithelial cells. *J. Cell Biol.* 103:1751-1765.
26. Nelson, W. J., and P. J. Veshnock. 1987. Modulation of membrane-skeleton (fodrin) stability by cell-cell contact in madin-darby canine kidney epithelial cells. *J. Cell Biol.* 104:1527-1537.
27. Nelson, W. J., and P. J. Veshnock. 1987. Ankyrin binding to the (Na^+ + K^+) ATPase and implications for the organization of membrane domains in polarized cells. *Nature (Lond.).* 328:533-535.
28. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.)* 308:693-698.
29. O'Keefe, E., T. Battin, and R. E. Payne, Jr. 1982. Epidermal growth factor in human epidermal cells: direct demonstration in cultured cells. *J. Invest. Dermatol.* 78:482-487.
30. O'Keefe, E., R. A. Briggaman, and B. Herman. 1987. Calcium induced assembly of adherens junctions in keratinocytes. *J. Cell Biol.* 105:807-817.
31. O'Keefe, E., R. E. Payne, Jr., and N. Russell. 1985. Keratinocyte growth-promoting activity from human placenta. *J. Cell Physiol.* 124:429-445.
32. Palfrey, H., and A. Waseem. 1985. Protein kinase C in the human erythrocyte. *J. Biol. Chem.* 260:16021-16029.
33. Repasky, E. A., B. L. Granger, and E. Lazarides. 1982. Widespread occurrence of avian spectrin in nonerythroid cells. *Cell.* 29:821-833.
34. Rheinwald, J., and H. Green. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature (Lond.).* 265:421-424.
35. Schliwa, M., T. Nakamura, K. R. Porres, and U. Enteneuer. 1984. A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J. Cell Biol.* 99:1045-1059.
36. Shen, B. W., R. Josephs, and T. L. Steck. 1984. Ultrastructure of unit fragments of the skeleton of the human erythrocyte membrane. *J. Cell Biol.* 99:810-821.
37. Srinivasan, Y., L. Elmer, J. Davis, V. Bennett, and K. Angelides. 1988.

Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature (Lond.)*. 333:177–180.
38. Takeichi, M. 1987. Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. *Trends Genet.* 3:213–217.
39. Watt, F., D. L. Mattey, and D. R. Garrod. 1984. Calcium induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell Biol.* 99:2211–2215.
40. Werth, D. K., and I. Pastan. 1984. Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. *J. Biol. Chem.* 259:5264–5270.
41. Wolfe, M., and A. Sayhoun. 1986. Protein kianse C and phosphatidylserine bind to M_r 110,000/115,000 polypeptides enriched in cytoskeletal and postsynaptic density preparations. *J. Biol. Chem.* 261:13327–13332.
42. Wulf, E., A. Deboben, F. A. Bautz, H. Faulstich, and T. Wieland. 1980. Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. USA*. 76:4498–4502.