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Polarisation-dependent association of plectin with desmoplakin and the lateral submembrane skeleton in MDCK cells

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SUMMARY

The intermediate filament-binding protein plectin and cytokeratin were localised at the cellular periphery of fully polarised Madin-Darby canine kidney (MDCK) cells, whereas vimentin was primarily found in a perinuclear network. Confocal and immunoelectron microscopy revealed that plectin was restricted to areas underlying the lateral plasma membrane. It colocalised with fodrin, a component of the submembrane skeleton, and was closely associated with desmosomal plaque structures. Biochemically, plectin was shown to interact directly with immunoprecipitated desmoplakin in vitro. Upon loss of cell polarity in low calcium medium, plectin redistributed to a cytoplasmic vimentin- and cytokeratin-related network, clearly distinct from diffusely distributed fodrin and internalised desmoplakin structures. The structural reorganisation of plectin was also reflected by an increased solubility of the protein in Triton X-100/high salt, and a decrease in its half-life from ~20 to ~5 hours. Furthermore, unlike cytokeratins and vimentin, desmoplakin and fodrin did not associate with plectin attached to magnetic beads in cell lysates of unpolarised cells, while all proteins formed a stable complex in polarised cells. Altogether, these data indicate that plectin is involved in the anchorage of intermediate filaments to desmosomes and to the submembrane skeleton in polarised MDCK cells.

Key words: Cytokeratin, Desmosome, Fodrin, Intermediate filament, Simple epithelium

INTRODUCTION

Simple epithelial cells represent a well characterised polarised cell type present in many tissues and organs. They contain two structurally and functionally distinct plasma membrane domains (apical and basolateral), which are at least in part established and maintained by cell-cell junctions and cell-substrate attachment sites at the basolateral membrane, and by a specialised cytoskeleton mostly associated with these junctional complexes (for recent reviews see Eaton and Simons, 1995; Drubin and Nelson, 1996). Intermediate filament (IF) networks are anchored at desmosomes and hemidesmosomes, two junctional complexes involved in cell-cell and cell-basal lamina interactions, respectively (for reviews see Schwarz et al., 1990; Garrod, 1993). They form a supracellular filamentous network that contributes to the structural integrity and mechanical strength of epithelial tissues. The molecular mechanisms leading to the establishment of an epithelial-specific IF system during cell differentiation are not yet known, but it seems very likely that proteins specifically interacting with IFs (for reviews see Foisner and Wiche, 1991; Fuchs and Weber, 1994), might be important regulators for the supramolecular IF network structure. One prominent and important group of IF-interacting proteins in epithelial cells mediates the anchorage of IFs to desmosomes. Although much has been learned about the constituents of desmosomal structures over the past few years, the precise interactions between desmosomes and IFs are still largely unknown (for review see Cowin and Burke, 1996). Desmoplakin I and II, splice variants of a widely expressed gene in stratified and simple epithelia, are possible candidates for mediating the IF/desmosome interaction, but the data available are controversial. Although the ectopically expressed carboxy-terminal domain of desmoplakin has been shown to coalign with and disrupt IF networks when expressed in non-polarised cells of simple epithelial origin (Stappenbeck and Green, 1992), the in-vitro binding studies failed to demonstrate a direct interaction of the bacterially expressed carboxy-terminal domain with simple epithelial type II keratins (Kouklis et al., 1994). Interestingly, the same assays revealed a binding of the desmoplakin domain to some basic keratins of the stratified skin epithelium. Other desmosomal proteins, which have been shown to bind to IFs in vitro, such as desmocalmin (Tsukita and Tsukita, 1985), band6 polypeptide (Kapprell et al., 1988; Hatzfeld et al., 1994), and a lamin B-related protein (Cartaud et al., 1990) seem to be restricted to certain stratified epithelia. Aside from the solely desmosomal proteins, most other epithelial IF-interacting proteins, which have been described to play important roles in the organisation of the epithelial IF system (Foisner and Wiche, 1991), are also restricted to stratified epithelia. Thus, unlike in stratified epithelia, very little is known about the molecular mechanisms involved in the formation of a supracellular IF network in polarised simple epithelial cells.

Plectin, a high M_r cytomatrix protein is a likely candidate

for an IF organising protein in simple epithelial cells, as it has been found to be abundantly expressed in a wide variety of tissues and cell types (for review see Wiche, 1989). In addition, plectin has been localised to the cellular periphery at cytoskeleton-membrane interaction sites in frozen sections of stratified skin epithelia and muscle tissues (Wiche et al., 1983, 1984; Foisner et al., 1994). At the biochemical level, it has been shown to interact with various cytoplasmic and nuclear IF subunit proteins (Foisner et al., 1988, 1991a), as well as with various other cytoskeletal proteins, including the microtubule-associated proteins MAP 1 and 2, and fodrin, a component of the submembrane skeleton (Herrmann and Wiche, 1987). Ultrastructural studies (Foisner and Wiche, 1987), and cDNA cloning and amino acid sequence analyses (Wiche et al., 1991; Liu et al., 1996) revealed a multi-domain organisation of the plectin molecule, consisting of an α-helical central coiled-coil structure flanked by two globular domains. The carboxy-terminal domain contains six highly homologous repeat structures which have been found in lesser numbers also in desmoplakin, in the hemidesmosome-associated protein bullous pemphigoid antigen (Green et al., 1992) and in the cornified envelope precursor protein envoplakin (Ruhrberg et al., 1996). Similar to desmoplakin, ectopically expressed plectin domains containing at least a 50 amino acid stretch within its carboxy-terminal repeat five domain were found to associate with IF structures in COS and Ptk_2 cells, and eventually caused the collapse of IF networks around the nucleus (Wiche et al., 1993; Nikolic et al., 1996).

To get a better understanding of plectin's potential role in the organisation of IF networks in polarised simple epithelial cells, we studied plectin's localisation and interactions in MDCK cells at different stages of polarisation. We show that plectin is primarily localised along the whole lateral plasma membrane of fully polarised cells, colocalising with fodrin, and with desmoplakin at sites of desmosomes, and redistributes to a cytoplasmic IF-type network upon loss of epithelial polarity. Furthermore, the association of plectin with a Triton X-100/high salt-insoluble cell fraction was significantly reduced upon loss of polarity, and desmoplakin and fodrin dissociated from the plectin/IF complex. For the first time, we also provide evidence that plectin might directly interact with desmoplakin and participate in the attachment of IFs at desmosomal structures in polarised simple epithelial cells.

MATERIALS AND METHODS

Cell culture

Madin-Darby canine kidney (MDCK) cells, strain II, were routinely cultivated on 100 mm plastic dishes (Falcon, Oxnard, CA) in high glucose DMEM, supplemented with 10% FCS, 10 mM Hepes, pH 7.2, 50 i.u./ml penicillin, 50 μg/ml streptomycin (all Life Technologies, Paisley, UK) at 37°C in a humidified atmosphere containing 5% CO_2.

Polarised cells were obtained by cultivating confluent cells for 4-6 days on 24 mm filter inserts (Transwell, Costar, Badhoevedorp, Netherlands), coated with Collagen type I (Collaborative Biomedical Products, Bedford, MA). To induce the loss of epithelial polarity, cells were cultivated in low calcium medium (LCM: S-MEM supplemented with non-essential amino acids (Life Technologies), 10% calcium-free FCS, 10 mM Hepes, pH 7.2, penicillin, streptomycin) for up to 24 hours.

Metabolic labelling

Polarised MDCK cells grown on filters were labelled in methionine-free DMEM medium containing 50 μCi/ml EXPRE [^{35}S]-protein labelling mix (1,175 Ci/mMol, Du Pont NEN, Bad Homburg, Germany) at the basolateral side for 4 hours. Desmoplakin was immunoprecipitated from cell lysates under high stringency conditions (see below) and analysed by SDS-PAGE and autoradiography. For the plectin turnover analyses, confluent cells grown in LCM or LCM plus 1.8 mM $CaCl_2$ (HCM) for 24 hours were incubated in methionine-free medium (LCM or HCM) containing 50 μCi/ml EXPRE [^{35}S]-protein labelling mix for 1 hour (pulse), and chased in a >10,000-fold excess of unlabelled methionine in the appropriate growth medium for 1 to 8 hours. Plectin was immunoprecipitated from cell lysates under high stringency conditions and analysed by SDS-PAGE and scintillation counting.

Antibodies

The following immunoreagents were used: monoclonal antibodies to plectin (Foisner et al., 1991b); antiserum to plectin (Wiche et al., 1983); antiserum raised against mouse mammary epithelial cell cytokeratins (Reichmann et al., 1992), kindly provided by H. Beug, IMP, Vienna; monoclonal antibody to vimentin (Dakopatts, Glostrup, Denmark); monoclonal antibodies to Desmoplakin I+II (Boehringer Mannheim, Germany; and Progen, Heidelberg, Germany); rabbit antiserum NW6 to desmoplakin I+II (Angst et al., 1990), kindly provided by K. Green, Northwestern University, Chicago, IL; the rat monoclonal antibody R40.76 to ZO-1 (Anderson et al., 1988), kindly provided by M. S. Mooseker, Yale University, CT; antiserum to α-fodrin (Nelson and Veshnock, 1986), kindly provided by W. J. Nelson, Stanford University, CA; secondary antibodies coupled to alkaline phosphatase (Promega, Madison, WI); secondary antibodies conjugated to Bodipy (Molecular Probes, Leiden, Netherlands), to Texas Red (Accurate Chemical & Scientific Corp., Westbury, NY), and to 5 nm or 20 nm gold particles (BioCell Res. Lab, Cardiff, UK).

Immunofluorescence and immunoelectron microscopy

Cells, grown on filters, were fixed for 3 minutes in methanol:acetone (1:1) at –20°C. Small pieces of the filters were blocked in PBS plus 0.2% gelatin for 30 minutes, incubated with the primary antibodies in PBS for 60 minutes, washed three times in PBS, incubated with the secondary antibodies for 60 minutes, washed in PBS, and mounted in Mowiol. Samples were viewed in a Zeiss Axiophot microscope, or with the MRC 600 confocal microscope (Bio-Rad, Hercules, CA).

For preembedding labelling immunoelectron microscopy, MDCK cells cultivated on filters were briefly rinsed in 200 mM Hepes, pH 7.4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), benzamidine (10 mM), approtinin, pepstatin, and leupeptin (each 10 μg/ml). After prefixation with 0.1% glutaraldehyde/0.1% Triton X-100 in Hepes buffer for 2 minutes at room temperature, and reduction of free aldehyde groups by three 5 minute incubations in $NaBH_4$ (0.5 mg/ml), cells were incubated with 0.1% Triton X-100 in Hepes buffer for 15 minutes and blocked for 30 minutes in 0.2% gelatin in Hepes buffer. For immunostaining, cells were incubated with a monoclonal antibody to plectin for 7 hours, washed several times, and incubated with a secondary antibody coupled to 5 nm gold particles for 7 hours. Postfixation was done for 30 minutes in 1% glutaraldehyde in Hepes buffer. After incubation in 1% OsO_4 in PBS for 1 hour, and several washes in PBS the samples were incubated in 1% uranyl acetate in 30% ethanol for 30 minutes, dehydrated in a graded series of ethanol, infiltrated with propylene oxide:Quetol (1:1 and 1:2) and embedded in Quetol. Ultrathin sections were cut with an ultramicrotome (Ultracut S, Reichert, Vienna), stained with uranyl acetate/lead citrate and viewed in a JEOL JEM-1210 transmission electron microscope at 80 kV.

For cryosectioning, cells were fixed with 2% formaldehyde in Hepes buffer for 45 minutes, embedded in 10% gelatin, and incubated in cryo-protectant overnight (Tokoyasu, 1986). The epithelial layer of

bovine tongue was peeled off, cut into small pieces, fixed in formaldehyde and incubated in cryo-protectant. Cryosectioning, and immunolabeling was performed as described by Griffiths et al. (1984), using antiserum to plectin and monoclonal antibody to desmoplakin followed by secondary antibodies coupled to 5 nm and 20 nm gold particles, respectively.

Subcellular fractionation and immunoprecipitation

MDCK cells from one 10 cm culture dish were lysed in 500 μl ice-cold 50 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 0.1 mM DTT, 0.5% Triton X-100, 0.5 mg/ml DNAse, 0.2 mg/ml RNAse (Boehringer Mannheim), and protease inhibitors. Cell lysates were either mixed with SDS-PAGE sample buffer (Laemmli, 1970) or adjusted to 1% Triton X-100 and 500 mM NaCl, and soluble and insoluble fractions were separated by centrifugation for 20 minutes at 14 krpm in a microfuge at 4°C, and dissolved in sample buffer.

For high stringency immunoprecipitation, immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF) and 1% SDS were added to the lysates or cell fractions to dissolve all protein complexes. After dilution with immunoprecipitation buffer to a final SDS concentration of 0.1%, the samples were processed for immunoprecipitation as described by Foisner et al. (1996), using monoclonal antibodies to plectin covalently coupled to CNBr-activated Sepharose beads (Pharmacia Biotech, Brussels, Belgium), or monoclonal antibody to desmoplakin and Protein G-Sepharose beads (Sigma, Deisenhofen, Germany).

Immunoisolation

For immunoisolation of plectin and associated proteins, total cell lysates (see above) containing soluble and finely dispersed insoluble protein complexes were diluted ten- to twentyfold in lysis buffer containing 1% Triton X-100 and 500 mM NaCl, and vigorously homogenised. Monoclonal antibodies to plectin conjugated to tosyl-activated magnetic beads (0.2 mg/ml, M-280, Dynal, Oslo, Norway) were added and incubated for 1 hour at room temperature. The beads were isolated in a strong magnetic field (MPC-M sample holder, Dynal), washed three times in the same buffer, dissolved in sample buffer and processed for SDS-PAGE and immunoblotting. Control experiments were performed using magnetic beads coupled to unspecific mouse immunoglobulins.

In vitro binding assay

Plectin was purified from polarised MDCK cells, using the protocol described previously (Foisner and Wiche, 1987). Desmoplakin was immunoprecipitated from MDCK cell lysates under high-stringency conditions to disrupt all protein complexes (see immunoprecipitation). 50-100 μl of desmoplakin immunoprecipitate and 500 μl of purified plectin (50-60 μg/ml) were mixed in binding buffer (PBS, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF), incubated for 1 hour at room temperature, and precipitated through a 30% sucrose cushion. The precipitate was washed in binding buffer and analysed by SDS-PAGE.

Other procedures

SDS-PAGE was performed according to the method of Laemmli (1970). Transfer of proteins onto nitrocellulose (0.2 μm, Schleicher & Schueller, Dassel Germany) was done in 40 mM glycine, 48 mM Tris, using the Bio-Rad Mini Trans-blot system. For the immunological detection of the proteins the Protoblot Immunoscreening System (Promega, Madison, WI) was used. Quantification of Coomassie-stained protein bands was done by densitometric scanning using a Hirschmann Elscript 400 densitometer, and bands in immunoblots were analysed using the NIH image software.

RESULTS

Distribution of plectin in polarised MDCK cells

To examine the localisation of plectin in polarised MDCK cell monolayers, confluent cells were cultured on porous filter supports for several days and then subjected to immunofluorescence and confocal microscopy. The peripheral localisation of uvomorulin (E-cadherin), a protein of adherens junctions (data not shown), and ZO-1 (Fig. 1), a marker protein of tight junctions, confirmed the establishment of a fully polarised phenotype with structurally intact adherens and tight junctions. In these fully polarised cells plectin was found to be located almost exclusively at the cellular periphery, giving rise to a strong and uniform staining along the whole circumference of the cells (Fig. 1). In a side view, generated by optically section-

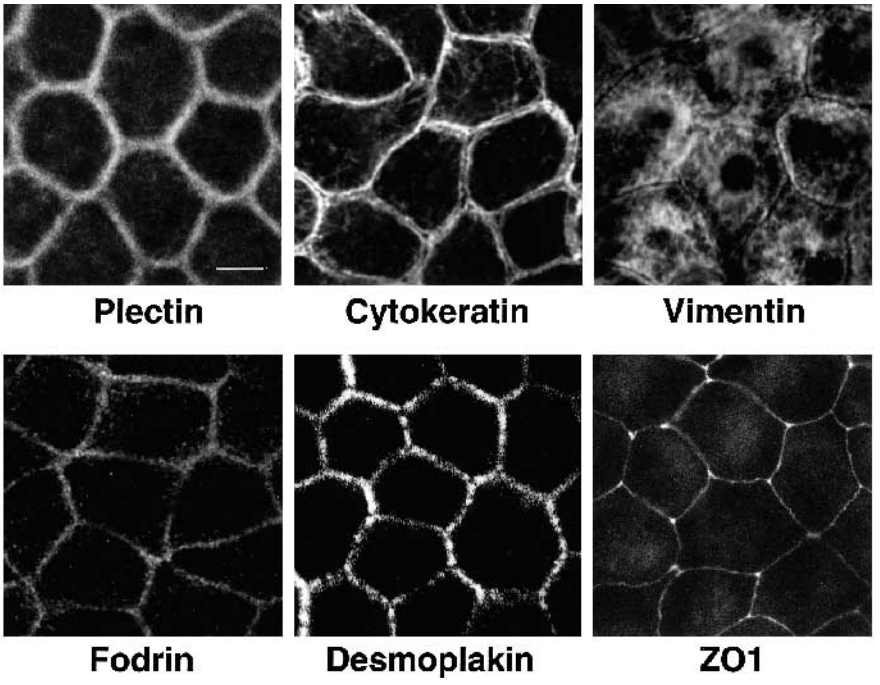

Fig. 1. Cellular localisation of cytoskeletal proteins in polarised MDCK cells. Cells were grown on porous filter supports until they reached the fully polarised state and processed for confocal microscopy. Antibodies used were: monoclonal antibody to plectin, antiserum to cytokeratin, monoclonal antibody to vimentin, antiserum to fodrin, monoclonal antibody to desmoplakin, and monoclonal antibody to ZO-1. Bar, 10 μm.

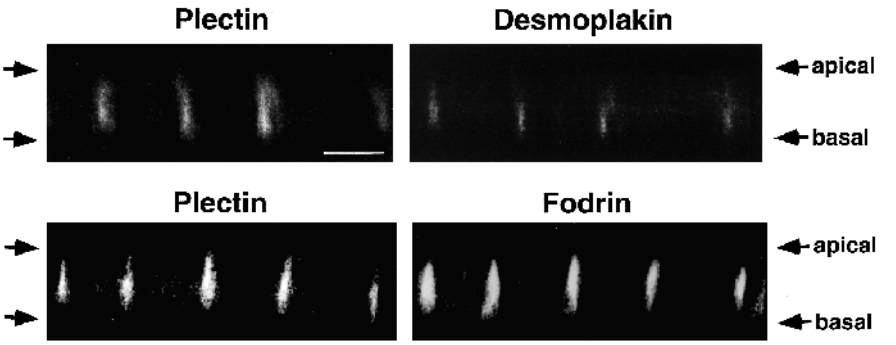

Fig. 2. Cellular localisation of plectin, desmoplakin and fodrin in vertical sections of polarised MDCK cells. Cells were grown on filter supports to confluency and double stained using antiserum to plectin and monoclonal antibody to desmoplakin (upper panel), or monoclonal antibody to plectin and antiserum to fodrin (lower panel). Optical sections in a plane perpendicular to the cell layer were generated with the confocal microscope. Bar, 10 μm.

ing the samples in a plane perpendicular to the cell layer, plectin was clearly shown to be restricted to regions along the lateral plasma membrane, whereas it was undetectable in the cytoplasm as well as along the basal and apical membrane domains (Fig. 2). The plectin staining colocalised primarily with that of the submembrane skeleton protein fodrin (Fig. 2), suggesting that plectin is associated with the cortical cytoskeleton at the lateral plasma membrane. Unlike plectin, desmoplakin was detected in distinct, spot-like structures along the membrane region in horizontal sections (Fig. 1), and was found at more basal regions of the lateral membrane in vertical sections (Fig. 2), reminiscent of proteins found in desmosomal plaque structures. Although the plectin staining apparently was more uniformly distributed along the entire lateral membrane, there was a clear overlap of desmoplakin-positive structures with those stained by the antibodies to plectin (Fig. 2), suggesting a close spatial association of plectin with the desmosomal plaque structures.

As for the organisation of the IF systems in the polarised phenotype, cytokeratin bundles were highly concentrated along the lateral plasma membrane, whereas vimentin was mostly located in a perinuclear network that was clearly distinct from the peripheral cytokeratin and plectin structures. (Fig. 1). There was a clear overlap of the plectin and cytokeratin structures at the cellular periphery, but, in contrast to plectin, the IF staining revealed clearly discernible unstained gaps between adjacent cells (Fig. 1). As plectin's localisation at these cytokeratin-free gaps was also confirmed by superimposing the staining patterns of both proteins (data not shown), it can be concluded that plectin is spatially more closely associated with the membrane than the cytokeratin filaments.

Ultrastructural localisation of plectin in polarised MDCK cells

To determine more precisely the localisation of plectin at the cell periphery we carried out preembedding immunogold labelling of polarised MDCK cells using monoclonal antibodies to plectin in conjunction with secondary antibodies coupled to 5 nm gold particles. As revealed by transmission electron microscopy of ultrathin sections, the polarised cells assumed a cylindrical morphology, formed an ordered, tightly packed monolayer, and exhibited apically located microvilli and intact junctional complexes (Fig. 3A; see also Nelson and Veshnock, 1986). At a closer inspection of the lateral cell-cell contact sites the plectin-specific gold label was found to be concentrated in close proximity to the lateral plasma membrane, but also extended some distance into the cytoplasm often associated with filamentous, probably cytokeratin, structures (Fig. 3B). Moreover, we frequently found the plectin label concentrated at the surface of desmosomal plaque regions (Fig. 3C). A statistical analysis of the distance of plectin-specific gold particles from the desmosomal membrane indicated that plectin was located between 50 and 125 nm from the membrane with an average distance of ~90 nm (Fig. 3G, upper panel). However, due to the preparation technique involving detergent extraction and preembedding labelling, membranes were often not clearly discernible and more peripheral plectin epitopes might be unaccessible for the antibodies. Therefore cryo-sectioning followed by post-embedding labelling was performed. This technique confirmed the tight association of plectin with the lateral plasma membrane (Fig. 3D) and the desmosomes (Fig. 3E), showing an overall distance of the plectin-specific label from the desmosomal plasma membrane similar to the preembedding labelling. As the low label densities in the cryo-sections did not allow accurate statistical analyses, we used bovine epithelial tissues, where the ultrastructural localisation of desmosomal components has previously been studied in detail (Miller et al., 1987; Skalli et al., 1994), and performed cryo-sectioning and post-embedding labelling using antibodies to plectin (small gold particles) and desmoplakin (large gold particles) (Fig. 3F). Morphometric analyses of these electron micrographs revealed that the localisation of plectin was similar to that obtained after preembedding labelling of MDCK cells (50-100 nm) and that, in agreement with previously reported studies (Miller et al., 1987; Skalli et al., 1994), desmoplakin was located at a distance between 25 and 50 nm from the desmosomal membrane (Fig. 3G). Considering the extraordinary length of individual plectin molecules (~200 nm, Foisner and Wiche, 1987; Foisner et al., 1995; Svitkina et al., 1996), the protein might function as a linker between IFs and desmoplakin.

In vitro interaction of plectin with desmoplakin

The previously reported in vitro interaction of plectin with fodrin (Herrmann and Wiche, 1987) is consistent with our structural analyses. Biochemical data on plectin's association with desmosomal proteins are still missing. Therefore, we performed in vitro binding studies using plectin purified from polarised MDCK cells and immunoprecipitated desmoplakin. To ensure that the immunoprecipitated desmoplakin did not contain contaminating proteins, polarised MDCK cells were first solubilized in a buffer containing 1% SDS to disrupt all protein complexes and, following dilution of SDS to 0.1%, desmoplakin was immunoprecipitated using a monoclonal antibody against desmoplakin and Protein G-Sepharose beads.

SDS-PAGE and autoradiography of desmoplakin samples immunoprecipitated from cell lysates of metabolically labelled cells revealed that they contained mainly desmoplakin I and were free of contaminating proteins (Fig. 4, lane DP). When desmoplakin, still bound to the beads, was incubated with purified plectin (Fig. 4, lane Pl.), centrifuged through a sucrose cushion, and analysed by immunoblotting, plectin was found to cosediment with immobilised desmoplakin (Fig. 4, lanes 2). Since control beads without desmoplakin (coated with the desmoplakin antibody alone) did not precipitate significant amounts of plectin (Fig. 4, Anti-Plectin, lane 3), it may be concluded that plectin interacted directly with desmoplakin. In addition, immunoblotting confirmed that cytokeratins, potential mediators of the plectin-desmoplakin interaction, were not detectable in the samples (Fig. 4, Anti-Cytokeratin).

Rearrangement of plectin structures and dynamics of plectin interactions upon loss of cell polarity

Calcium deprivation from the culture medium of polarised epithelial cell cultures has previously been shown to cause the loss of epithelial polarity, the internalisation of junctional plaques, and gross perturbations of the cytoskeleton and the submembrane

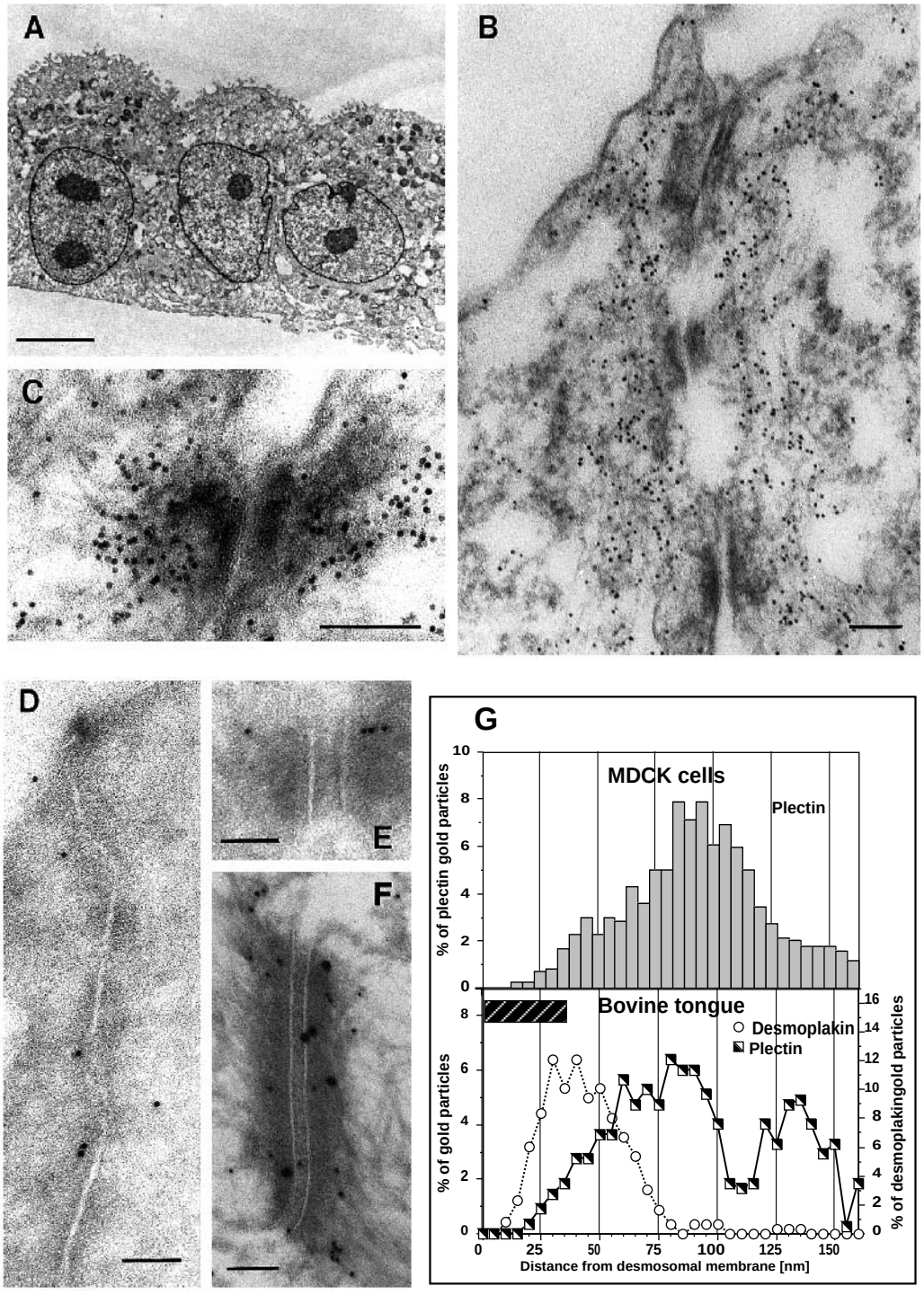

Fig. 3. Ultrastructural localisation of plectin and desmoplakin. MDCK cells were grown on filter supports to confluency and processed for immunoelectron microscopy according to the preembedding labelling technique using monoclonal antibodies to plectin (A-C) or the cryosectioning/post-embedding labelling technique using antiserum to plectin (D,E). Low magnification image of cell layer (A); and higher magnification images of a lateral cell-cell contact site (B,D) and of a desmosome (C,E) are shown. (F) Cryo-section of a bovine tongue epithelial desmosome labelled according to the post-embedding labelling technique using antibodies to plectin (small gold particles) and desmoplakin (large gold particles). Bars: 5 μm (A); 100 nm (B-F). (G) Statistical analyses of the distance of plectin-specific gold particles from the plasma membrane at desmosomal plaque regions in MDCK cells revealed from images as shown in C, and of plectin and desmoplakin in bovine tongue revealed from images shown in F. In comparison, the position of the majority of desmoplakin (95%) in bovine nasal epithelial desmosomes (see Miller et al., 1987) is indicated by the horizontal bar.

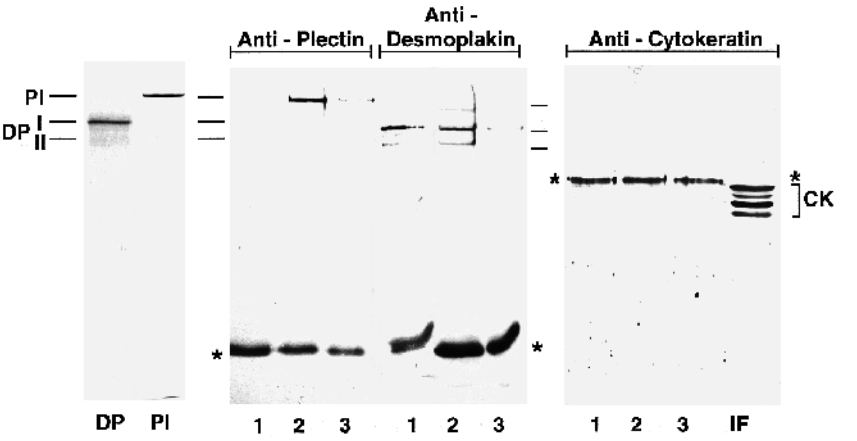

Fig. 4. In vitro interaction of plectin with desmoplakin. Desmoplakin was immunoprecipitated from polarised MDCK cells using monoclonal antibodies to desmoplakin and Protein G-Sepharose beads. For binding studies, immunoprecipitated desmoplakin alone (lanes 1), or immunoprecipitated desmoplakin mixed with purified plectin (lanes 2), or control beads without desmoplakin (coated with the monoclonal desmoplakin antibody alone) mixed with purified plectin (lanes 3) were centrifuged through a sucrose cushion, and pellets were analysed by immunoblotting using monoclonal antibodies to plectin, antiserum to desmoplakin and antiserum to cytokeratin. Lane DP, autoradiogram of desmoplakin immunoprecipitated from metabolically labelled cells; lane Pl, Coomassie-stained purified plectin; lane IF, IF-preparation from MDCK cells; stars, immunoglobulin heavy chain of the desmoplakin antibody. Note that, unlike the immunoprecipitation, immunoblotting was performed with antiserum to desmoplakin, which cross-reacted slightly with plectin.

skeleton (Nelson and Veshnock, 1986; Duden and Franke, 1988; Pasdar and Nelson, 1988a,b; Kartenbeck et al., 1991). To gain further insights into the epithelial-specific interactions of plectin, and to analyse the effects of the loss of polarity on its cellular localisation, fully polarised cells were cultivated in low calcium medium (LCM, Ca^{2+}<10 μM) for various time periods up to 24 hours and analysed by immunofluorescence microscopy. Within 4 hours of cultivation in LCM, plectin and cytokeratin gradually retracted from the cell periphery and the number of cytokeratin bundles extending to cell-cell contact points steadily decreased (Fig. 5A, 4h LCM). After 24 hours cultivation in LCM the cells have lost their cell-cell contacts, and plectin was found throughout the cytoplasm, largely colocalising with the cytokeratin (Fig. 5A, 24h) and the perinuclear vimentin network (data not shown). Vertical sections revealed that after 2-4 hours of Ca^{2+}-deprivation plectin-positive structures were only partially concentrated at the

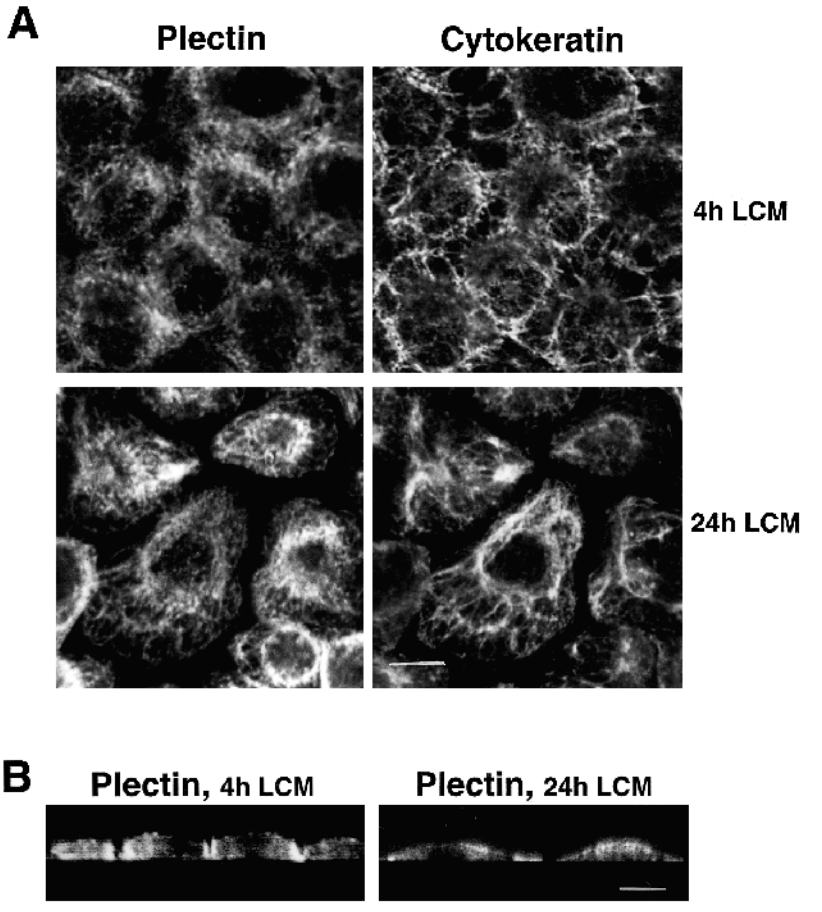

Fig. 5. Structural reorganisation of plectin and cytokeratin upon loss of epithelial polarity. Polarised MDCK cells grown on filter supports were incubated in low calcium medium (LCM) for 4 and 24 hours and processed for confocal microscopy using monoclonal antibodies to plectin and antiserum to cytokeratin. Horizontal optical sections of double stained images (A) and vertical images of single stained samples are shown. Bar, 10 μm.

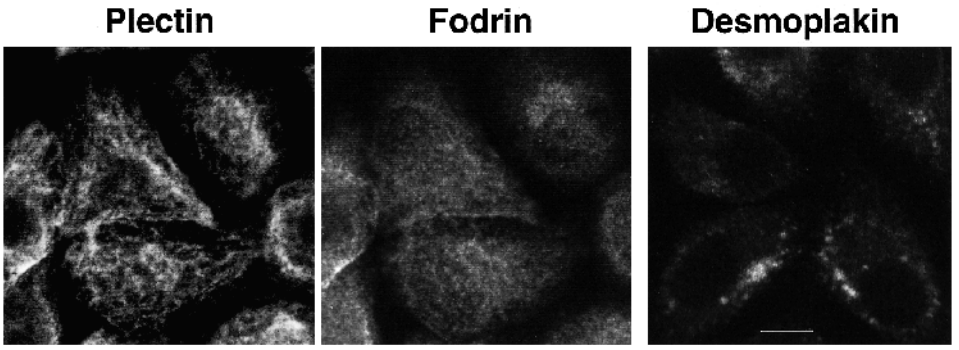

Fig. 6. Cellular localisation of plectin, desmoplakin and fodrin in unpolarised cells. Polarised MDCK cells grown on filters were cultivated for 24 hours in LCM and processed for confocal microscopy using monoclonal antibody to plectin, antiserum to fodrin and monoclonal antibody to desmoplakin. The plectin and fodrin images represent a double staining. Bar, 10 μm.

lateral membrane and began to extend into the cytoplasm, while after 24 hours they were distributed throughout the cytoplasm in the flattened and well spread cells (Fig. 5B). Fodrin was also redistributed into the cytoplasm at the fully unpolarised stage, exhibiting a diffuse, but largely plectin-independent staining throughout the cell (Fig. 6). Desmoplakin has been completely internalised into granular, probably vesicular structures (Duden and Franke, 1988; Dehmlehner et al., 1995), and did not show significant colocalisation with plectin structures (Fig. 6). Hence, plectin's association with the submembrane skeleton and desmosomes was dependent on a polarised phenotype.

To examine the changes in plectin's association with cytoskeletal and junctional components at the biochemical level, fully polarised or unpolarised cells (following a 24 hour incubation in LCM) were lysed in buffers containing 1% Triton X-100 and 500 mM salt, and the soluble and insoluble fractions were analysed by immunoblotting. As demonstrated in Fig. 7A, vimentin and cytokeratin IFs were largely insoluble in both polarised and unpolarised cells. The majority of plectin and desmoplakin cofractionated with the insoluble IF-containing fraction in polarised cells (Fig. 7A, HCM), whereas more than half of the total amount of these proteins was shifted into the soluble fraction upon loss of polarity (Fig. 7A, lanes LCM). A statistical analysis of these data revealed that the relative amount of soluble plectin increased from 15-25% to 40-60% upon cultivation of the cells in LCM for 24 hours (Fig. 7B). Kinetic studies showed that as early as 15 minutes after calcium removal an increase in the solubility of plectin could be detected and reached a maximum level within 2-3 hours, staying constant for up to 24 hours (Fig. 7C). Taken together, it may be concluded that in polarised cells plectin and desmoplakin are components of highly stable, insoluble cytoskeletal structures which are profoundly rearranged upon loss of polarity.

To examine the polarity-dependent changes in the interactions of plectin with IF proteins, desmoplakin and fodrin in more detail, we performed co-immunoisolation studies using monoclonal antibodies to plectin covalently coupled to magnetic beads. Antibody-coated beads were added to highly diluted total cell

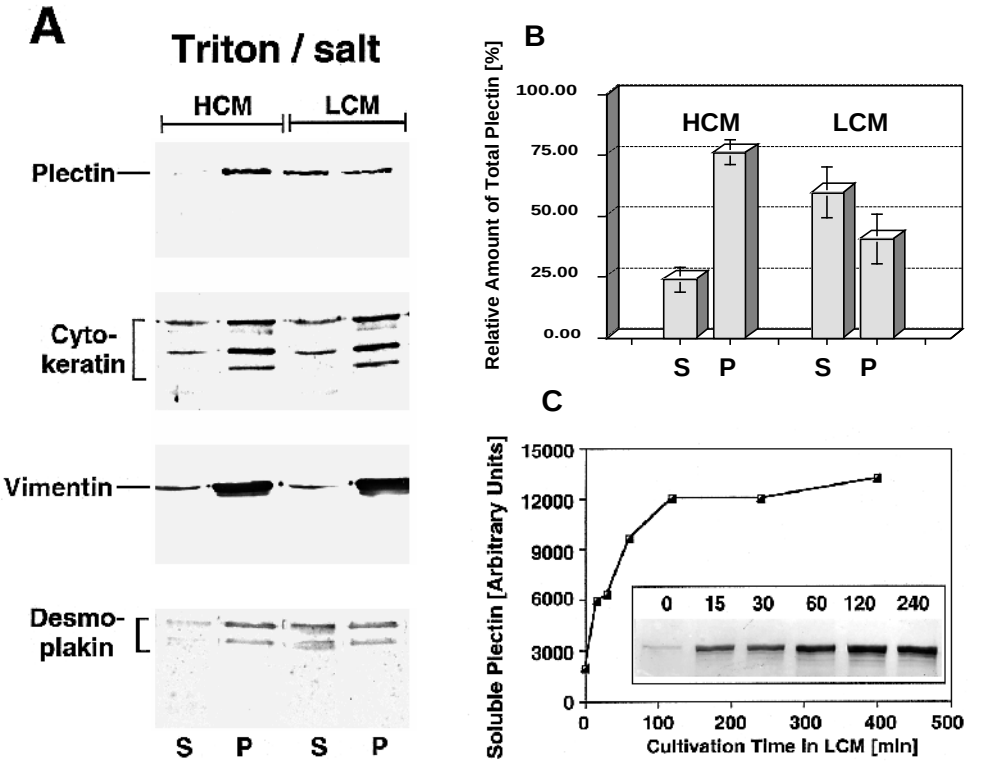

Fig. 7. Subcellular fractionation of cytoskeletal components in polarised and unpolarised MDCK cells. Polarised MDCK cells grown in high calcium medium (HCM) or unpolarised cells grown in low calcium medium (LCM) for 24 hours were lysed in buffers containing 1% Triton X-100 and 500 mM salt.
(A) Immunoblot analysis of soluble (S) and insoluble (P) fractions. Note that loaded HCM and LCM samples do not contain exactly identical amounts of total proteins, but supernatant and pellet fractions represent identical cell equivalents. (B) Quantification of the relative amount of plectin in fractions of polarised (HCM) and unpolarised (LCM) cells. Values represent means of 5 independent experiments.
(C) Kinetics of the increase in solubility of plectin in 1% Triton X-100/500 mM salt upon cultivation in LCM for various time points, determined by densitometric scanning of plectin bands in immunoblots of soluble cell fractions (insert).

lysates of polarised and unpolarised cells, and the beads together with the proteins specifically associated with the beads were isolated in a magnetic field and analysed by immunoblotting. Unlike control beads coated with unspecific mouse IgGs (data not shown), the beads coated with the plectin antibody pulled out similar amounts of plectin, vimentin and cytokeratin from polarised and unpolarised cells (Fig. 8). Desmoplakin and fodrin, on the other hand, were found to associate with the beads only in samples of polarised cells, whereas samples of unpolarised cells (Fig 8, left panel) or samples obtained with the control beads (data not shown) did not contain significant amounts of these proteins. As the cell lysates from polarised and unpolarised cells were shown by immunoblotting to contain similar amounts of desmoplakin and fodrin (Fig. 8 right panel), both proteins dissociated from the IF-plectin network upon loss of cell polarity.

Metabolic stability of plectin in polarised versus unpolarised MDCK cells

The structural rearrangements of desmoplakin and fodrin upon loss of polarity have been found to be accompanied by a significant decrease in the metabolic stability of these proteins (Nelson and Veshnock, 1986, 1987; Pasdar and Nelson, 1988a). To determine whether loss of polarity affects the metabolic stability of newly synthesised plectin in a similar way, polarised (HCM-medium) and unpolarised (LCM-medium for 24 hours) MDCK cells were pulse-labelled for 1 hour using [^{35}S]methionine and chased for up to 8 hours in the respective medium (LCM or HCM). Plectin was immunoprecipitated from cell lysates, and the label incorporated per μg of plectin was determined. As indicated by a decrease in the calculated half-life from 18-24 hours in polarised to 4-8 hours in unpolarized cells (Fig. 9), the turnover rate of newly synthesised plectin was significantly increased upon loss of polarity. Despite this significant decrease in the half life of plectin, we did not observe a change in the total amount of the protein (see Fig. 8), probably due to the relative short time period tested (24 hours). However, the increased degradation might also be counteracted by an increased synthesis rate, considering that the incorporated label was nearly twofold higher in unpolarised (LCM) versus polarised (HCM) cells (Fig. 9A).

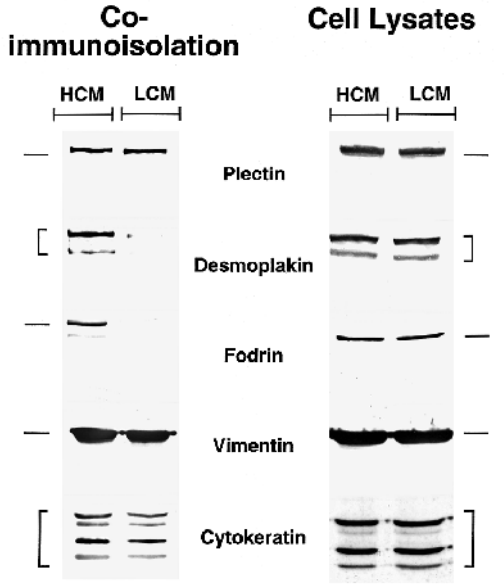

Fig. 8. Association of desmoplakin and fodrin with the plectin-IF complex in polarised versus unpolarised cells. Total cell lysates of polarised (HCM) and unpolarised (LCM) MDCK cells, were analysed by immunoblotting (right panels, Cell lysates) or incubated with magnetic beads coupled to monoclonal antibodies to plectin, and following isolation of magnetic beads in a magnetic field, proteins specifically associated with the beads were analysed by immunoblotting (left panels, Co-immunoisolation).

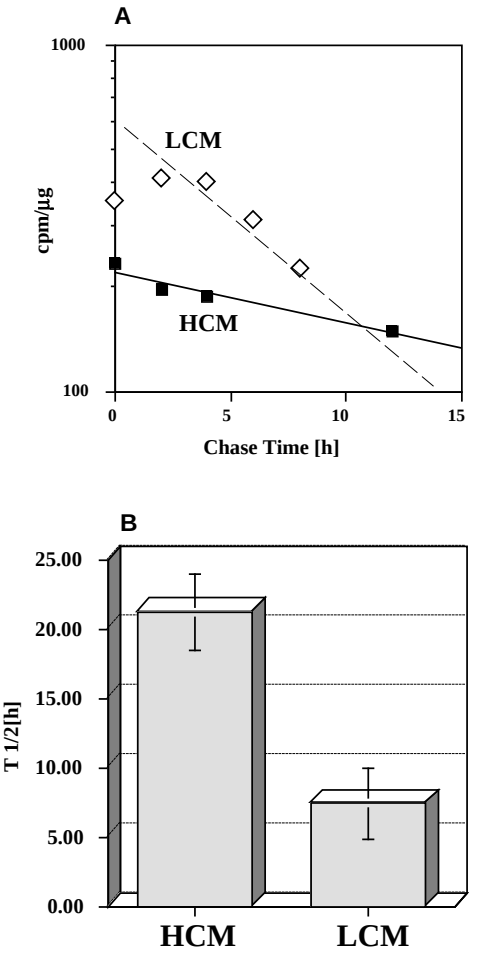

Fig. 9. Rate of turnover of newly synthesised plectin in polarised and unpolarised MDCK cells. Polarised (HCM) and unpolarised (LCM) cells were metabolically labelled using [^{35}S]methionine and chased for indicated time periods in medium containing cold methionine. Plectin was immunoprecipitated from cell lysates, analysed by SDS-PAGE, and the radioactive label incorporated per μg of plectin was determined by scintillation counting and densitometric scanning of Coomassie-stained plectin bands. (A) Decrease of plectin-specific label of one particular experiment shown in a half logarithmic scale; (B) Calculated half life of plectin. Error bars represent s.d. calculated from 3 independent experiments.

DISCUSSION

Although a number of IF-interacting proteins have been described in stratified epithelia, and have been implicated in the anchorage of IFs at the membrane (Schwarz et al., 1990; Fuchs and Weber, 1994), very little is known about the molecular inter-

actions of IFs in polarised simple epithelial cells. To address the role of the IF-interacting protein plectin in the establishment of an IF system specific for polarised epithelia, we used a model cell system (MDCK), allowing the formation of a single cell sheet of fully polarised simple epithelial cells in culture. We found that plectin was predominantly localised underneath the lateral plasma membrane in fully polarised cells, and was barely or not at all detectable at the basal and apical plasma membrane and throughout the cytoplasm. Plectin structures clearly overlapped with cytokeratin bundles at the cellular periphery, but obviously reached farther outwards to areas underlying the lateral plasma membrane. At these sites, plectin was found to colocalise with fodrin, a component of the submembrane skeleton, which has been suggested to be involved in the establishment of cell polarity in MDCK cells (Nelson and Veshnock, 1987; Nelson and Hammerton, 1989). These findings, together with previous studies, revealing a direct binding of fodrin to plectin in vitro (Herrmann and Wiche, 1987), and an association of transiently expressed plectin proteins with simple epithelial keratins in cultured cells (Wiche et al., 1993; Nikolic et al., 1996), indicated that plectin is involved in both the structural organisation of the peripheral cytokeratin bundles and their association with the submembrane skeleton.

Using immunoelectron microscopy, plectin was also detected at the cytoplasmic surface of desmosomal dense plaque regions. The ultrastructural localisation of plectin at desmosomes was reminiscent of the cellular localisation of pinin, identified in simple and stratified epithelia by means of a monoclonal antibody (Ouyang and Sugrue, 1996), and of the plectin-related protein IFAP-300 in certain stratified epithelia (Skalli et al., 1994). As these proteins have been suggested to be involved in the stabilisation of the IF-desmosome complexes or to link IFs to the desmosomes, it is tempting to speculate that plectin might function in a similar way in polarised simple epithelial cells. This notion is further supported by our in vitro binding data, showing a direct association of plectin with the desmosomal plaque protein desmoplakin.

The association of plectin with non IF structures, such as desmosomes and the submembrane skeleton, was dependent on the polarised state of the cells. Upon loss of cell polarity induced by calcium deprivation, the distribution of plectin changed from a mostly peripheral localisation to an IF-related cytoplasmic network that was clearly different from the internalised desmoplakin and fodrin structures. Furthermore, immobilised plectin antibodies pulled out desmoplakin and fodrin together with plectin and IFs only from cell lysates of polarised cells, whereas these proteins were absent in samples from unpolarised cells. Although these co-immunoisolation studies did not prove a direct binding of plectin to desmoplakin and fodrin, they provided evidence that plectin and cytokeratin together with desmoplakin and fodrin formed a stable complex that was dependent on the existence of functional cell-cell junctions and of a lateral submembrane skeleton. Further evidence for plectin's differential interactions in polarised versus unpolarized cells was provided by the observation that the solubility of plectin in Triton X-100/high salt was significantly increased upon loss of epithelial polarity. As the solubility of desmoplakin (Pasdar and Nelson, 1988b, and shown here) and fodrin (Nelson and Veshnock, 1986), unlike that of IF proteins, increased in a similar way upon loss of polarity, it may be concluded that in polarised cells the association of plectin with non-IF structures, such as desmosomes and the submembrane skeleton, made plectin more resistant towards chemical extraction.

The differential cellular distribution and associations of plectin, desmoplakin, fodrin and IFs in polarised versus unpolarised cells raises the question as to how this reorganisation might be regulated. Phosphorylation of desmoplakin at a specific site in its carboxy terminus has been implicated in the regulation of the desmoplakin-IF interaction (Stappenbeck et al., 1994). Plectin has been found to serve as a substrate for various kinases, including protein kinases A and C, Ca^{2+}/calmodulin-dependent kinase, and $p34^{cdc2}$ kinase (Herrmann and Wiche, 1983, 1987; Foisner et al., 1991a, 1996; Malecz et al., 1996). As our previous studies have indicated that the phosphorylation of plectin by protein kinase C and $p34^{cdc2}$ kinase modulated its interactions with IFs in non epithelial cells (Foisner et al., 1991a, 1996), it is conceivable that phosphorylation might also be responsible for the regulation of plectin's interactions in epithelial cells.

The potential of plectin to link IFs to junctional complexes does not seem to be restricted to simple epithelia. Previous data on plectin's localisation in various tissues (Wiche et al., 1983, 1984; Foisner et al., 1994) and immunoelectron microscopy of bovine tongue epithelium shown here indicate that plectin might fulfil similar functions in stratified epithelia and in muscle tissues. The role of plectin in linking IFs to hemidesmosomes in skin tissue has recently been underlined by the identification of homozygous frameshift mutations and loss of plectin expression in an autosomal recessive disease, characterised by skin blistering and late-onset muscular dystrophy (MD-EBS) (Gache et al., 1996; Smith et al., 1996). Altogether, plectin is very likely to represent an essential component for the formation of a supracellular epithelial IF-network that confers mechanical stability and integrity to polarised simple epithelial cells as well as to stratified epithelial tissues.

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