

IFAP 300 Is Common to Desmosomes and Hemidesmosomes and Is a Possible Linker of Intermediate Filaments to These Junctions

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Abstract. The distribution of IFAP 300, a protein previously characterized as cross-linking vimentin intermediate filaments (IF), has been investigated in epithelial cells. In frozen sections of bovine tongue epithelium the staining obtained with IFAP 300 antibodies is concentrated in the peripheral cytoplasm of keratinocytes, including the entire peripheral region of basal cells. Further immunofluorescence studies reveal that in primary cultures of mouse keratinocytes the distribution of IFAP 300 is similar to that of the desmosomal protein desmoplakin. In rat bladder carcinoma 804G cells the staining pattern of IFAP 300 antibodies coincides with that obtained with antibodies against the hemidesmosomal protein BP 230. By immunogold electron microscopy IFAP 300 is mainly located at sites where IF appear to attach to desmosomes and hemidesmosomes. Morphometric analyses of the distribution of the gold particles show that IFAP 300 overlaps with desmoplakin and BP 230, but also that it extends deeper into the cytoplasm than these latter two proteins. The staining reaction seen in epithelial cells by immunofluorescence and immunogold

is specific for IFAP 300 as shown by immunoblotting. Immunoblotting also reveals that IFAP 300 is present in both cell-free preparations of desmosomes and hemidesmosomes. These morphological and biochemical results are intriguing since, in recent years, the proteins appearing in these two types of junctions have been found to be different. One possible exception is plectin, a protein that has been suggested to be very similar to IFAP 300. However, we show here that IFAP 300 differs from plectin in several respects, including differences at the primary sequence level. We also show that purified IFAP 300 pellets with *in vitro* polymerized IF prepared from desmosome-associated keratins under conditions in which IFAP 300 alone is not sedimentable. This indicates that IFAP 300 can associate with keratin IF. These data, taken together with the immunogold results, suggest that IFAP 300 functions in epithelial cells as a linker protein connecting IF to desmosomes as well as to hemidesmosomes, possibly through structurally related proteins such as desmoplakin and BP 230, respectively.

THE overall organization of intermediate filaments (IFs)¹ is similar in most cell types, consisting of a filamentous network that extends from the nucleus to the cell surface (Goldman et al., 1986). However, during differentiation this network is often modified to some extent to fulfill cellular or tissue-specific functions (for review see Traub, 1985). Frequently, these modifications include changes in the association of IF with different cellular components, among which are specialized plasma membrane domains involved in cell-substratum or cell-cell adhesion such as hemidesmosomes and desmosomes (for reviews see

Schwarz et al., 1990; Jones and Green, 1991; Garrod, 1993). The interaction of IF with these junctional complexes leads to the formation of a supracellular filamentous network, the integrity of which is critical for at least some of the mechanical properties of epithelial tissues (Bonifas et al., 1991; Coulombe et al., 1991a,b; Lane et al., 1992). These possible mechanical functions are most dramatically supported by severe epidermal blistering in some human diseases (Bonifas et al., 1991; Coulombe et al., 1991b; Lane et al., 1992) and in transgenic animals (Coulombe et al., 1991a; Vassar et al., 1991) in which the keratin IF network is disrupted because of the expression of mutant keratin genes.

At sites of desmosomes and hemidesmosomes, ultrastructural studies have suggested that IF are anchored to the inner plaque located at the cytoplasmic face of a larger dense plaque lining the plasma membrane (for review see Staehelin, 1974). Despite this similar ultrastructural organization, to date no protein has been convincingly shown to be a common component of desmosomes and hemidesmosomes

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1. *Abbreviations used in this paper:* BP, bullous pemphigoid; IF, intermediate filaments; IFAP, IF-associated protein; PME, primary mouse epithelial; TAME, N α -p-tosyl-L-arginine methyl ester.

(Jones et al., 1986; for reviews see Schwarz et al., 1990; Jones and Green, 1991; Garrod, 1993). This raises the possibility that the molecule(s) involved in linking IF to desmosomes are different from those linking IF to hemidesmosomes. Although several proteins have been associated with the binding of IF to desmosomes or hemidesmosomes (for reviews see Schwarz et al., 1990; Jones and Green, 1991; Garrod, 1993), there is currently no direct evidence regarding which proteins actually link IF to these junctions.

An interesting observation pertaining to this problem is that antibodies against plectin stain epithelial tissues in a pattern suggesting an association with both desmosomes and hemidesmosomes (Wiche et al., 1983, 1984). Since plectin has been defined as an IF-associated protein (IFAP) (Foisner and Wiche, 1991) this observation raises the possibility that IFAPs might be involved in linking IF to desmosomes and hemidesmosomes. A protein of apparent molecular mass similar to that of plectin (300 kD) and with similar binding properties to vimentin IF has been described in BHK fibroblasts and called IFAP 300 (Lieska et al., 1985; Yang et al., 1985). The comparison of IFAP 300 and plectin by various biochemical techniques has led to the suggestion that the two proteins are closely related (Herrmann and Wiche, 1987), although differences in the staining pattern of antibodies directed against each of these proteins have been reported (Wiche and Baker, 1982; Yang et al., 1985). Since the primary sequence of plectin is now known (Wiche et al., 1991) and several antibodies are available which are directed against IFAP 300 or plectin, it is now possible to carry out direct comparisons between plectin and IFAP 300. Preliminary data relative to this issue are reported in this paper and point toward significant differences between the two proteins.

In fibroblasts, IFAP 300 has been shown to colocalize with vimentin IF both during interphase (Yang et al., 1985) and in mitosis (Skalli et al., 1992). Neither the presence nor the subcellular localization of this protein has been described in epithelial cells. This is the goal of our present study. Our results show that in epithelial tissue and cultured epithelial cells IFAP 300 is concentrated at desmosomes and hemidesmosomes. We also show that IFAP 300 is present in enriched fractions of desmosomes and hemidesmosomes and that it binds to keratin IF *in vitro*. These data suggest that IFAP 300 is part of a common mechanism involved in the attachment of IF to desmosomes and hemidesmosomes.

Materials and Methods

Cell Culture

Primary mouse epidermal (PME) cells were obtained from neonatal BALB/c mice which were 36-h old using the method of Yuspa and Harris (1974). The cells were grown for four days in MEM containing low (0.02 mM) calcium, 10% calcium-free FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. To induce the formation of desmosomes CaCl_2 was added to a final concentration of 1.2 mM for 3–4 h (Hennings et al., 1980; Jones and Goldman, 1985). Rat bladder carcinoma 804G cells, BHK fibroblasts, and C6 glioma cells were grown as previously described (Pytela and Wiche, 1980; Yang et al., 1985; Riddelle et al., 1991).

Antibodies

A mouse monoclonal antibody and a rabbit antiserum directed against IFAP 300 were used in these studies (Yang et al., 1985). The monoclonal was used for the immunofluorescence and immunogold studies. To label desmo-

somes, we used a rabbit polyclonal (Jones et al., 1987) or a mouse monoclonal (ICN Immunobiologicals, Costa Mesa, CA) antibody directed against desmoplakin. To label hemidesmosomes, we used the human monoclonal antibody 5E-HY-4B directed against BP 230 (a gift of Dr. Hashimoto of Keio University School of Medicine, Department of Dermatology, Shinjuku, Tokyo, Japan) (Hashimoto et al., 1993). The monoclonal antibodies 1D8 and 10F6 directed against plectin were kind gifts of Dr. Gerhard Wiche (University of Vienna, Institute of Biochemistry, Vienna, Austria) (Foisner et al., 1991). Secondary antibodies for immunofluorescence or immunoblotting were purchased from Kirkegaard & Perry Labs., Inc. (Gaithersburg, MD). These antibodies were raised in goats against IgGs of the appropriate species and were conjugated to fluorescein or rhodamine for immunofluorescence or to peroxidase for immunoblotting. Secondary antibodies for immunogold were purchased from Ted Pella Inc. (Redding, CA). These antibodies were raised in goat against IgGs of the appropriate species and were conjugated to 5- or 10-nm gold particles.

Immunofluorescence

4- μ m-thick cryostat sections of bovine tongue frozen in liquid nitrogen were fixed with acetone for 5 min at -20°C and then air dried. PME and 804 G cells grown on coverslips were briefly rinsed in PBS (6 mM phosphate, 170 mM NaCl, 3 mM KCl, pH 7.4), fixed in -20°C methanol for 5 min and air dried.

The dilution of the primary antibodies was 1:5 for culture supernatants containing monoclonal antibodies and 1:100 for polyclonal antibodies. Secondary antibodies were diluted 1:20. Incubation times for the primary and secondary antibodies were 30 min at 37°C . Each incubation was followed by three 5-min washes in PBS. The cells or tissue sections were mounted in gelvatol containing 0.1% diazabicyclo[2.2.2]octane.

Confocal microscope observations were carried out on a Zeiss LSM microscope (Carl Zeiss Inc., Oberkochen, FRG) using a He/Ne laser at 514 nm for rhodamine and an Ar laser at 488 nm for fluorescein. A Zeiss Axiohot microscope equipped with epi-illumination and specific filters for fluorescein and rhodamine was used for nonconfocal microscopic observations. Photographs were taken on Kodak Plus-X black and white films.

Immunogold Electron Microscopy

Double immunogold localization of IFAP 300 and desmoplakin or of IFAP 300 and BP 230 was performed on 5–8- μ m-thick cryostat sections of bovine tongue fixed for 5 min in -20°C acetone. The sections were incubated overnight with the appropriate combination of primary antibodies diluted 1:10 for monoclonal antibodies or 1:50 for polyclonal antibodies. Following through washes in PBS, the sections were incubated for 30 min in normal goat serum diluted 1:20 in 20 mM tris/HCl, pH 8.2, 140 mM NaCl containing 1% BSA. After several washes in PBS, incubation with the appropriate combination of gold conjugate diluted 1:20 was carried out for 6 h. Following extensive washing in PBS, the sections were fixed in 1% glutaraldehyde, postfix in 1% OsO_4 and further processed for electron microscopy as described in Klatte et al. (1989). Electron microscopic observations were carried out with a JEOL 100CX (JEOL USA, Peabody, MA) electron microscope at an accelerating voltage of 60 kV.

To determine the distance of the gold particles to the plasma membrane, thin sections of desmosomes or hemidesmosomes taken perpendicular to the plasma membrane were photographed. Slides were made from these pictures and were projected to achieve a final magnification of 400,000 or 800,000. Distances were measured from the gold particle to the outermost part of the desmosomal- or hemidesmosomal-dense plaque. Following conversion of this distance into nanometers, an additional 10 nm was added to find the distance of the gold particle to the outer surface of the plasma membrane. This 10-nm distance corresponds to the width of the space that separates the dense plaque from the plasma membrane (Staehelin, 1974). Since $\sim 13\%$ of the gold particles had a size intermediate between 5 and 10 nm, we made measurements only on particles of unambiguous size.

We carried out these morphometrical analyses for 18 desmosomes, and for a total of 368 and 160 gold particles corresponding to the staining for IFAP 300 and desmoplakin, respectively. Similar measurements were carried out on 32 hemidesmosomes, for a total of 293 and 150 gold particles corresponding to the staining for IFAP and BP 230, respectively.

Preparation of Enriched Fractions of Desmosomes and Hemidesmosomes, and of BHK and C6 Cell IF-enriched Cytoskeletal Preparations

For all biochemical procedures the following protease inhibitors were in-

cluded in each buffer: 1 mM PMSF, 1 mg/ml TAME, 0.5 mg/ml aprotinin, 0.7 mg/ml leupeptin, and 0.5 mg/ml pepstatin.

Desmosome and hemidesmosome fractions were obtained from bovine tongue epithelium. To avoid proteolysis, fresh tongue epithelium was immediately removed from the connective tissue without soaking the mucosa overnight in 2 mM EDTA as is usually done (Skerrow and Maltotsy, 1974). To obtain desmosomes we followed the citrate extraction procedure of Skerrow and Maltotsy (1974) as modified by Tsukita and Tsukita (1985). To obtain a preparation enriched in hemidesmosomal components we followed the procedure of Klatter et al. (1989).

Cytoskeletal preparation of BHK fibroblasts or C6 glioma cells were prepared using the procedures of Starger et al. (1978) and Pytela and Wiche (1980), respectively.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed using the procedure of Laemmli (1974). The percentage of acrylamide was 6.5% in order to obtain an optimal separation of the high molecular mass (300 kD) proteins in which we were most interested. Blotting of the proteins separated by SDS-PAGE onto nitrocellulose membranes was done electrophoretically for 3 h at 100 V and at 4°C (Towbin, 1985). Nonspecific protein-binding sites on the nitrocellulose membrane were subsequently blocked with PBS containing 5% milk. The antibodies were diluted in this solution at 1:500 for the monoclonal antibodies, 1:1,000 for the polyclonal antibodies, and 1:2,500 for the peroxidase-conjugated secondary antibodies. Nitrocellulose-bound secondary antibodies were detected by chemiluminescence using the ECL kit (Amersham Corp., Arlington Heights, IL).

Radioiodination of Proteins, Two-dimensional Thin Layer Electrophoresis, and Chromatography

Gel slices containing IFAP 300 and plectin were obtained from SDS-polyacrylamide gels loaded, respectively, with IF-enriched cytoskeletal preparations from BHK fibroblasts and C6 glioma cells. The identity of the bands was confirmed by their reactivity with IFAP 300 or plectin antibodies. Furthermore, two-dimensional gel electrophoresis showed that there was a single major high molecular mass protein migrating at 300 kD in each of these preparations (not shown) (Herrmann and Wiche, 1987). The proteins were labeled following the protocol described by Judd (1990), using 100 μ Ci of 125 I and Iodobeads (Pierce Co., Rockford, IL) as an oxidizing agent. Digestion with trypsin was carried out overnight at 37°C by incubating the gel slices in 0.5 ml of ammonium bicarbonate containing 10 μ g of trypsin. The eluted peptides were recovered by lyophilization. 100,000 cpm of each sample were loaded on thin-layer cellulose plates (Kodak) and the tryptic peptides were separated by high voltage electrophoresis followed by ascending chromatography (Judd, 1990). 125 I-labeled peptides were identified by autoradiography on Kodak X-Omat film.

Purification Procedures for Desmosomal Keratins and IFAP 300

Desmosomal keratins were purified from bovine tongue desmosomes obtained as described above. Desmosomes were extracted for 1–2 h at room temperature in 9 M urea, 10 mM Tris/HCl, pH 9.0, 0.2% β -mercaptoethanol. The extract was ultracentrifuged at 100,000 g for 30 min and the supernatant was concentrated by precipitation with 6 vol of ethanol at –20°C. The precipitated proteins were collected by centrifugation at 10,000 g for 30 min and dissolved in 10 ml of 9 M urea, 10 mM Tris/HCl, pH 7.4, 0.2% β -mercaptoethanol containing the protease inhibitor mixture described above. Following dialysis against that buffer, the proteins were separated by gel filtration on a 100 \times 1.5-cm column packed with Sephacryl S-400 (bed volume 190 ml). The flow rate was 5 ml/h and 1-ml fractions were collected. The proteins present in every five fractions were analyzed on 7.5% SDS-polyacrylamide gels and those containing the keratin were identified by immunoblotting and were subsequently pooled.

IFAP 300 was purified from BHK-21 cells by gel filtration on Sephacryl S-400 as described by Lieska et al. (1985).

Polymerization of Desmosomal Keratins and Binding Assays with IFAP 300

To examine their ability to form IF, the keratins purified from bovine tongue desmosomes by gel filtration were diluted to a concentration of 0.5 mg/ml and dialyzed against 100 vol of assembly buffer (10 mM Tris/HCl, pH 7.4, 0.2% β -mercaptoethanol, and 1 mM PMSF). These preparations were ex-

amined for the presence of IF by electron microscopy after negative staining with 1% aqueous uranyl acetate.

For the binding assays, the desmosomal keratins were polymerized at a concentration of 0.5 mg/ml. IFAP 300 was maintained at a concentration of 0.5 mg/ml in the same buffer used to polymerize the keratins. In a typical experiment, 5 μ g of IFAP 300 was incubated with 50 μ g keratin IF for 2 h at room temperature. The proteins were collected by ultracentrifugation at 100,000 g for 30 min and the resulting pellets were analyzed by SDS-PAGE. As a control experiment, we examined whether or not IFAP 300 binds to myosin, which like keratin possesses an alpha helical domain. Rabbit skeletal myosin in solution in 50% glycerol, 10 mM phosphate buffer, pH 7.5, 0.6 M KCl was purchased from Sigma (St. Louis, MO). Myosin filaments were assembled by dialyzing the protein solution against PBS. The binding of IFAP 300 to myosin filaments was examined as described above for IFAP 300 and keratin.

Screening of a BHK Cell's λ gt11 cDNA Library, and Isolation and Sequencing of a Partial cDNA Clone Coding for IFAP 300

Approximately 10^6 plaques from a λ gt11 cDNA library of BHK cells (Sambrook et al., 1989) were screened with the monoclonal antibody directed against IFAP 300 using the protocol of Young and Davis (1985). Four positive plaques were selected and rescreened until a plaque-pure population was obtained. Fusion proteins were made from large scale cultures of Y1090 bacteria made lysogenic with the selected phages (Huynh et al., 1985). Mice were immunized with fusion protein bands cut from an SDS-polyacrylamide gel. After three injections the sera were tested by immunoblotting (above) against BHK cells total proteins and BHK cytoskeletal preparations. One of the four fusion proteins used as immunogen elicited the production of antibodies specific for IFAP 300. The cDNA insert coding for that protein was isolated and subcloned in pBluescript II KS (Stratagen Co., La Jolla, CA). The insert was sequenced using the dideoxy chain termination method (Sanger et al., 1977). Comparison of the sequence obtained with that of plectin was done using the Bestfit program of the Genetic Computer Group (University of Wisconsin Genetic Computer Group, Madison, WI).

Results

Desmosomes and Hemidesmosomes Are Stained by IFAP 300 Antibodies

The distribution of IFAP 300 in cryostat sections of tongue epithelium was studied by immunofluorescence. The results show that IFAP 300 antibodies stain intensely all surfaces of the stratified squamous epithelial cells (Fig. 1). In thinner sections, the distribution of IFAP 300 appears punctate at the lateral and upper surfaces of basal cells and at all cell surfaces of suprabasal cells. At these cell surfaces the staining pattern is very similar to that seen with desmoplakin antibodies (Jones et al., 1986). In the case of the basal surface of basal cells the staining patterns seen with anti-IFAP 300 and with antibodies against BP 230 (a hemidesmosomal marker) are coincident and appear as parallel, discontinuous lines (Fig. 1).

The localization of IFAP 300 was also determined in PME cells grown in medium containing 1.2 mM calcium to stimulate the formation of desmosomes (Hennings et al., 1980; Jones and Goldman, 1985). Under these conditions, IFAP 300 localizes primarily in areas of cell–cell contact, giving a punctate pattern (Fig. 2). In addition, some spots and diffuse fluorescence are also observed in the cytoplasm. However, the free edges of cells not in contact with other cells do not stain with IFAP 300. The staining pattern of anti-IFAP 300 at the cell periphery follows closely that of desmoplakin as shown by double immunofluorescence (Fig. 2). However, the staining region often appears broader than that

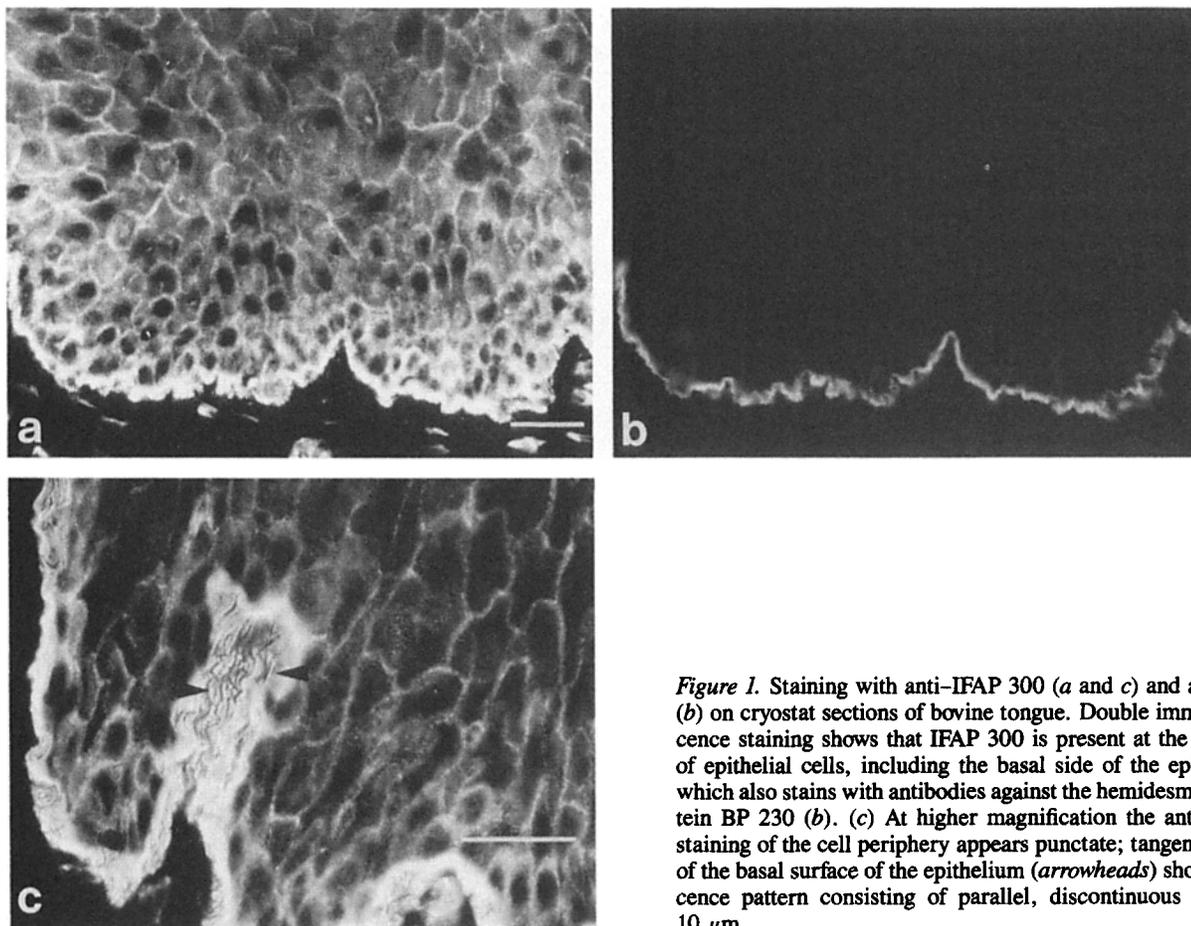


Figure 1. Staining with anti-IFAP 300 (*a* and *c*) and anti-BP 230 (*b*) on cryostat sections of bovine tongue. Double immunofluorescence staining shows that IFAP 300 is present at the cell surface of epithelial cells, including the basal side of the epithelium (*a*) which also stains with antibodies against the hemidesmosomal protein BP 230 (*b*). (*c*) At higher magnification the anti-IFAP 300 staining of the cell periphery appears punctate; tangential sections of the basal surface of the epithelium (*arrowheads*) show a fluorescence pattern consisting of parallel, discontinuous lines. Bars, 10 μm .

seen with desmoplakin antibodies (Fig. 2). No obvious staining of the IF network is seen in these cells.

We have also examined the relationship between IFAP 300 and hemidesmosomes in rat bladder carcinoma cells (804G line) which express numerous hemidesmosomes (Riddelle et al., 1991). In these cells, anti-IFAP 300 staining produces a pattern of bright spots of varying size and shape distributed in the region of cell-substrate-interactions as determined by confocal microscopy (Fig. 3). These spots are frequently clustered into ring-like structures surrounding regions devoid of stain. Identical staining is seen with anti-BP 230 demonstrating that these spots represent hemidesmosomes (Fig. 3). In these cells, the cytoplasmic IF network does not stain with anti-IFAP 300 (Fig. 3).

Immunogold Localization of IFAP 300

To determine more precisely the localization of IFAP 300 in desmosomes and hemidesmosomes, we carried out double label immunogold electron microscopy on frozen sections of bovine tongue epithelium. Secondary antibodies labeled with 5-nm gold particles were used to determine the localization of IFAP 300 and 10-nm gold particles were used to locate desmoplakin and BP 230. The localization of desmoplakin and BP 230 provides internal controls since their distributions have been well characterized at the ultrastructural level (Mueller and Franke, 1983; Jones and Goldman, 1985; Shimizu et al., 1985; Jones et al., 1986; Steinberg et

al., 1986; Miller et al., 1987; Klatte et al., 1989; Owaribe et al., 1990; Tanaka et al., 1990).

The gold particles corresponding to IFAP 300 antibodies are concentrated at sites of desmosomes and hemidesmosomes (Fig. 4). Gold particles corresponding to desmoplakin and BP 230 antibodies are located, as expected, in the inner plaque region of desmosomes and hemidesmosomes, respectively. The localization of the gold particles corresponding to desmoplakin or BP 230 is similar to that of the IFAP 300 antibodies. However, the latter frequently extend much further into the cytoplasm relative to desmoplakin or BP 230, and are associated with IF (Fig. 4).

Measurements of the distance from the plasma membrane to the gold particles indicate that desmoplakin and BP 230 are distributed in a very similar fashion (Fig. 5). The gold particles are located between 30–90 nm from the plasma membrane, and the average distance is about 50–55 nm. These values are consistent with previous findings (Jones et al., 1986; Steinberg et al., 1986; Miller et al., 1987). The first gold particles seen for anti-IFAP 300 are located at a distance of 40–50 nm from the plasma membrane. This distance corresponds to the location of the maximum number of gold particles labeling desmoplakin in desmosomes, or BP 230 in hemidesmosomes. The overlap between the distribution of IFAP 300 and these two latter antigens continues for approximately an additional 40 nm, to reach a distance of 80–90 nm from the plasma membrane. Distal to this latter

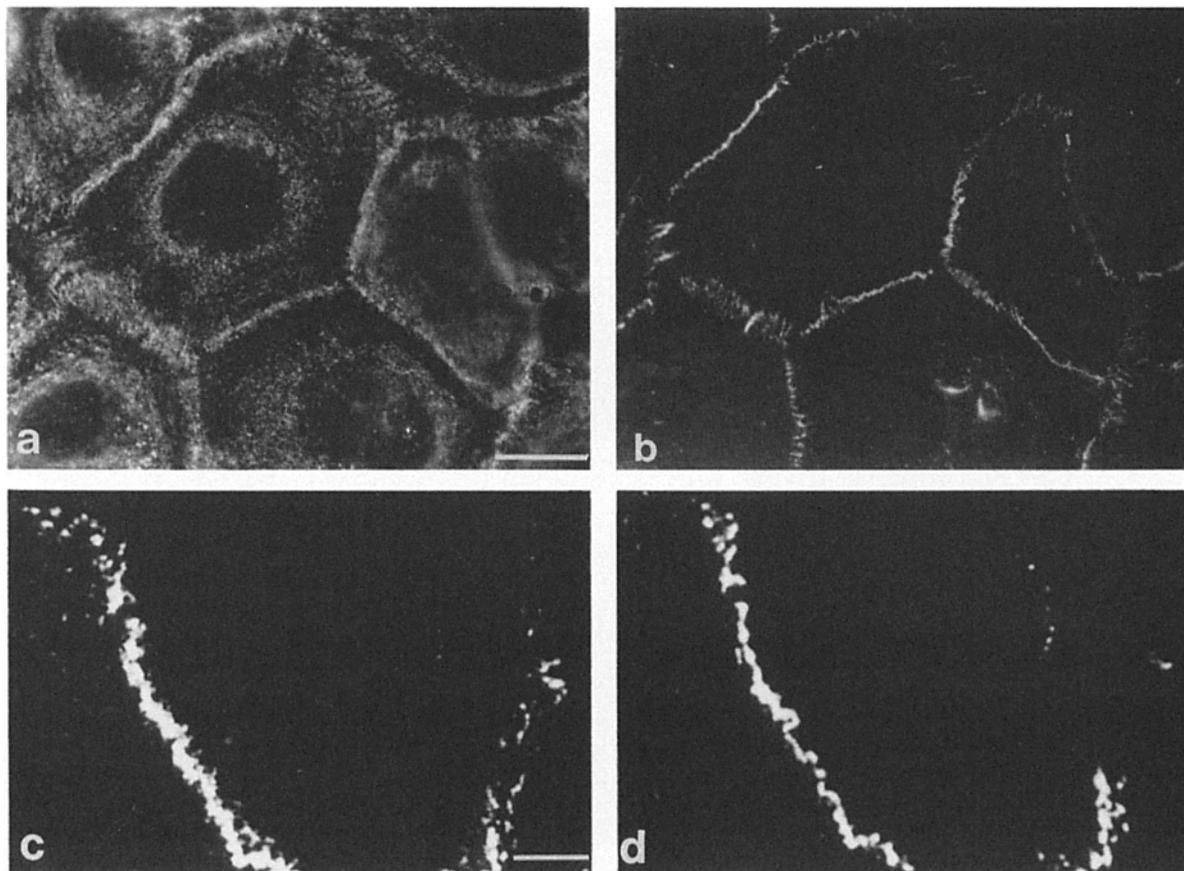


Figure 2. Double immunofluorescence of PME cells with anti-IFAP 300 (*a* and *c*) and desmoplakin (*b* and *d*). An overall view (*a* and *b*) shows the codistribution of IFAP 300 and desmoplakin at areas of cell-cell contact. In addition, with anti-IFAP 300 a diffuse or granular intracytoplasmic staining is also observed. Because of this intracellular staining, the colocalization of IFAP 300 with desmoplakin at sites of cell-cell contact was examined by confocal microscopy (*c* and *d*). This method emphasizes the colocalization and shows that the staining of IFAP 300 often appears broader than that of desmoplakin. Bars: (*a*) 10 μm ; (*c*) 1.5 μm .

submembranous region, the number of gold particles corresponding to IFAP 300 decreases gradually over a distance of an additional 100 nm. Few gold particles are seen beyond this distance from the plasma membrane. It should be noted that these measurements probably do not represent the actual distance from the antigen to the plasma membrane; rather they provide a rough estimation of relative distances of the different antigens. This is due to the fact that the gold particles are localized at a distance from the antigen which is related to the size of the intervening primary and secondary antibodies (e.g., Steinberg et al., 1986).

Specificity of IFAP 300 Antibodies and Presence of IFAP 300 in Preparations Enriched in Desmosomal or Hemidesmosomal Proteins

Desmoplakin and keratin have been shown to share epitopes (Gigi-Leitner and Geiger, 1986) which may result from the structure and sequence similarities that exist between these proteins (Green et al., 1992). In addition, it is known that BP 230, desmoplakin, and keratin contain domains with similar sequences (Green et al., 1990, 1992; Tanaka et al., 1991). In light of these observations, it is conceivable that IFAP 300 antibodies may cross-react with proteins such as desmoplakin and/or BP 230 known to be present in desmo-

somes or hemidesmosomes, rather than with bona fide IFAP 300. Therefore, we have carried out immunoblotting analyses to determine whether IFAP 300 is present in cell-free preparations of desmosomes and hemidesmosomes.

SDS-polyacrylamide gels demonstrate that isolated bovine tongue desmosomes contain a high molecular mass protein that comigrates with IFAP 300 prepared from BHK cells (Fig. 6). By immunoblotting, this 300-kD desmosomal polypeptide reacts with our monoclonal anti-IFAP 300 (Fig. 6). Similar assays carried out on bovine tongue preparations enriched in hemidesmosomal proteins demonstrate that these preparations also contain a 300-kD band recognized by the monoclonal antibody against IFAP 300 (Fig. 6).

IFAP 300 Binds to Desmosomal Keratin IF

Taken together, the ultrastructural localization of IFAP 300 at sites where IF are closely associated with the inner plaque of desmosomes and hemidesmosomes and the previous finding (Lieska et al., 1985) that IFAP 300 binds to vimentin IF, suggests that this protein may function in attaching IF to desmosomes and hemidesmosomes. This has prompted us to determine whether IFAP 300 can bind to keratin IF. For these experiments we have used the keratins that coisolate

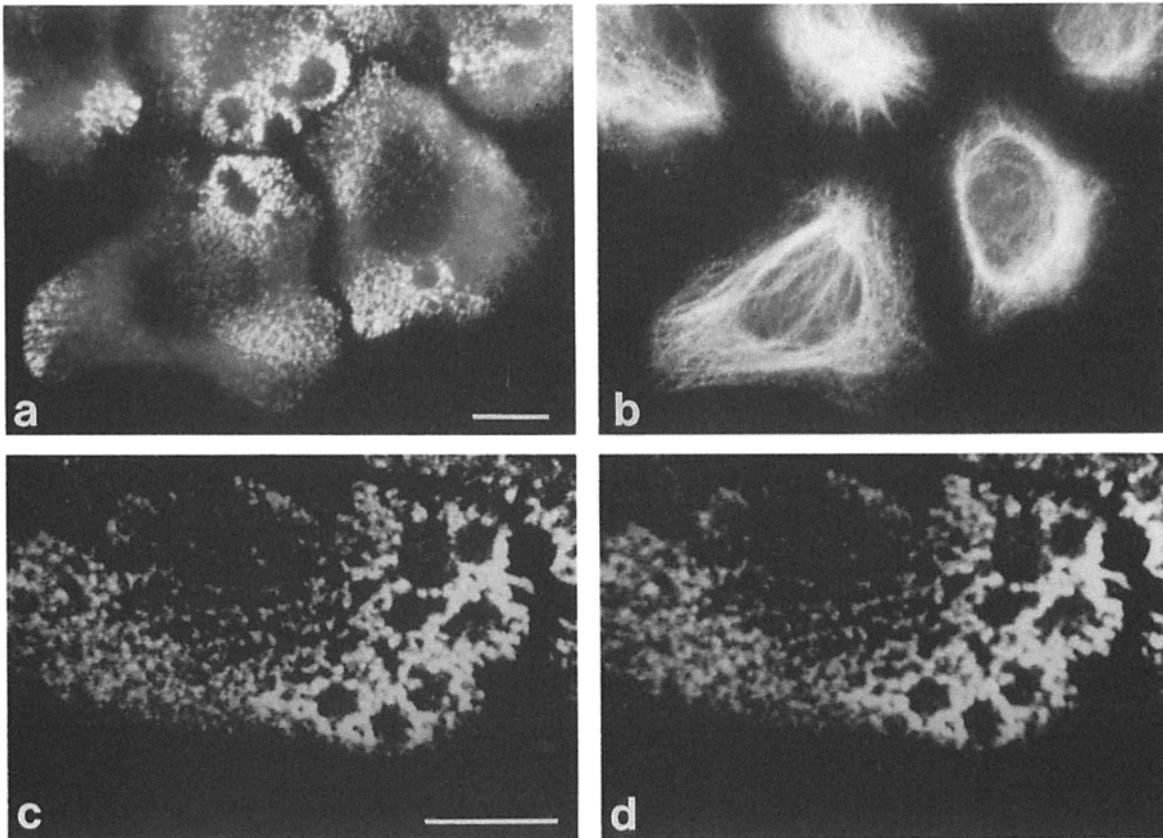
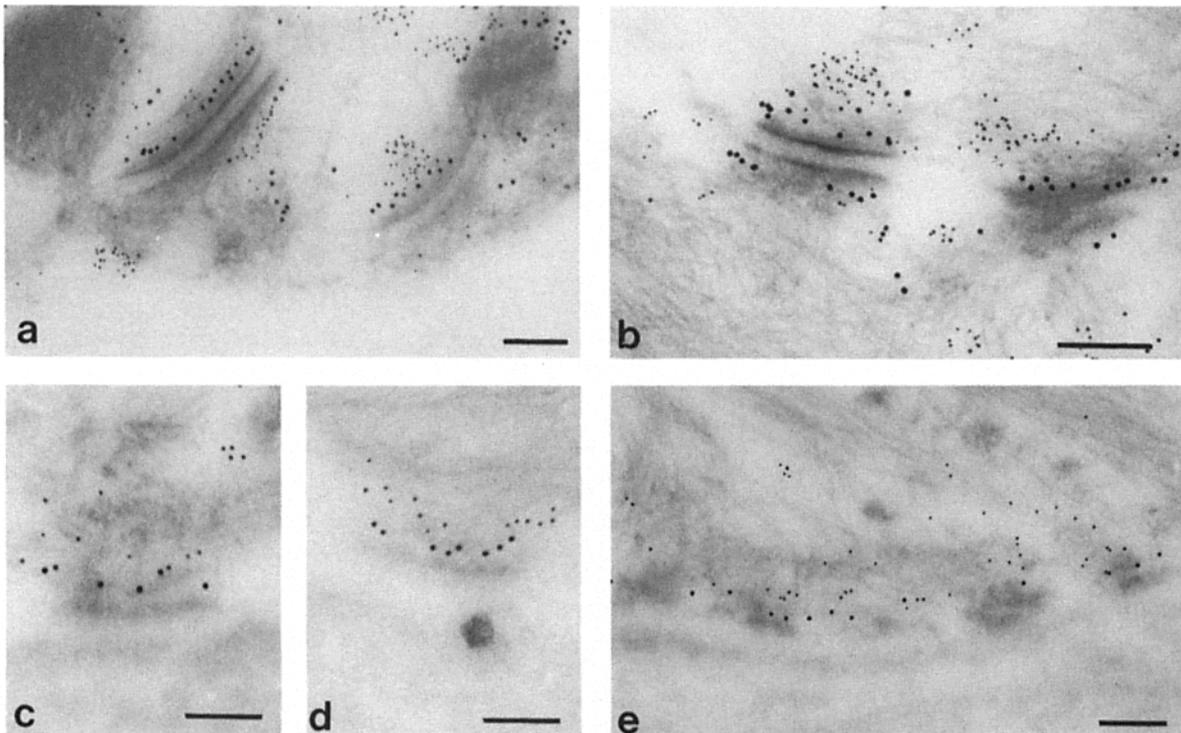


Figure 3. Double immunofluorescence staining of 804G cells with anti-IFAP 300 (*a*) and anti-keratin (*b*); and with anti-IFAP 300 (*c*) and anti-BP 230 (*d*). Anti-IFAP 300 shows arrays of spots often concentrated in discrete areas. IFAP 300 staining is distinct from that obtained with keratin antibodies (*b*), but is coincident with the staining obtained with BP 230 antibodies (*d*), as seen by confocal microscopy (*c* and *d*). In the confocal microscope the cells are only in focus in regions of cell substrate contact and not in the cytoplasmic or upper cell surface regions. Bars, 2.5 μm .

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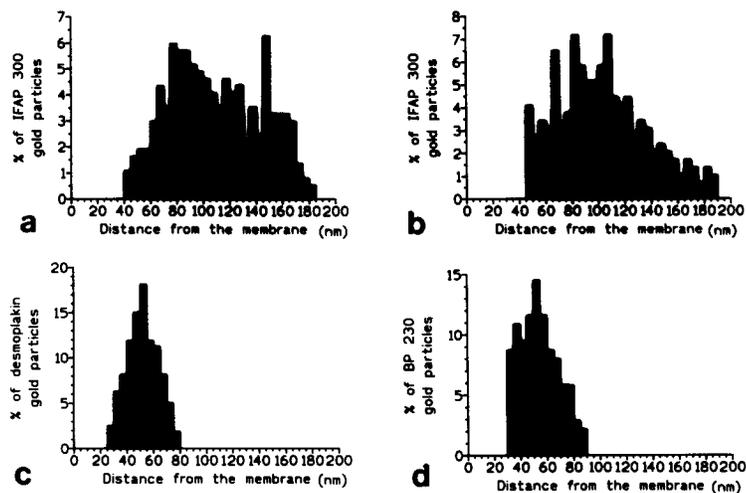


Figure 5. Histograms of the distribution of the 5-nm gold particles labeling IFAP 300 (*a* and *b*), and of the 10-nm gold particles labeling desmoplakin (*c*) and BP 230 (*d*). (*a* and *c*) Distribution over desmosomes, (*b* and *d*) distribution over hemidesmosomes. For a given antigen, each bar represents the percentage of gold particles present over a 5-nm interval. Distances are measured from the plasma membrane as described in Materials and Methods.

with desmosomes because they are concentrated at the site of IF association.

For the preparation of desmosome-associated keratins, freshly isolated bovine desmosomes are extracted in 8 M urea, a procedure known to solubilize the nonglycosylated desmosomal proteins (Franke et al., 1983; Jones et al., 1988). The urea extract is then fractionated by gel filtration on Sephacryl S-400. This is effective in separating the keratins from the other urea-soluble desmosomal proteins, including the desmoplakins. The protein compositions of the fractions are then analyzed by SDS-PAGE (Fig. 7) and only those greatly enriched for keratins are pooled. Following this procedure, the purity of the keratins is greater than 90%. These keratins consist of five polypeptides of about 64, 57,

52, 50, and 43 kD. In agreement with previous data from this laboratory, these polypeptides react with several keratin antibodies and appear identical in molecular weight and in relative proportions to those present in the tongue epithelium (Jones et al., 1988). We have also confirmed that the dialysis of the unfractionated urea-soluble desmosomal fraction against a keratin assembly buffer does not yield any recognizable IF (Jones et al., 1988). In contrast, under the same conditions of dialysis, the keratins pooled from the S-400 column readily form 10-nm filaments as observed by elec-

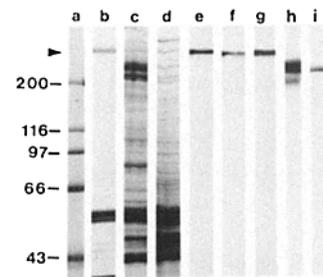


Figure 6. (*a-d*) Coomassie blue-stained 6.5% SDS-polyacrylamide gel of: (*a*) molecular mass markers (molecular mass is indicated in kilodaltons, arrow indicates the position of IFAP 300); (*b*) BHK cytoskeletal preparation; (*c*) desmosomes purified from bovine tongue epithelium; and (*d*) preparation enriched in hemidesmosomal proteins. Note the presence in *b* and *c* of a 300-kD polypeptide. By immunoblotting (*e-g*) this polypeptide reacts with a monoclonal antibody against IFAP 300 in the BHK cytoskeletal preparation (*e*), in bovine tongue desmosomes (*f*), and in a preparation enriched in hemidesmosomal proteins (*g*). Control immunoblot experiments with desmoplakin (*h*) and BP 230 (*i*) antibodies confirm the enrichment for desmoplakin in isolated desmosomes (*h*) and for BP 230 in preparations enriched in hemidesmosomes (*i*).

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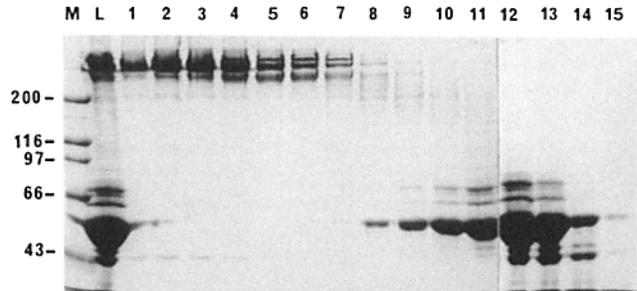


Figure 7. Coomassie blue-stained 7.5% SDS-polyacrylamide gel showing the purification of desmosomal keratin proteins from bovine tongue by gel filtration on Sephacryl S-400. (Lane *M*) Molecular mass markers (molecular mass is indicated in kilodaltons). (Lane *L*) Column load which is constituted by the urea-soluble desmosomal proteins. (Lanes 1-15) Protein profile of every other five fractions eluted after the bed volume. The first peak of proteins eluted from the column (lanes 1-7) contains mostly the desmoplakins. The second peak of proteins eluted from the column (lanes 8-15) contains mostly the keratin proteins. Only the fractions containing the least contamination by proteins other than keratin (fractions 14 and 15) were pooled.

Figure 4. Immunogold localization of IFAP 300 in desmosomes (*a* and *b*) and hemidesmosomes (*c-e*). Small (5-nm diam) gold particles were used to localize IFAP 300 (*a-e*), and large (10-nm diam) gold particles were used to localize desmoplakin in desmosomes (*a* and *b*) and BP 230 in hemidesmosomes (*c-e*). The distribution of the small gold particles shows the association of IFAP 300 with desmosomes (*a* and *b*) and hemidesmosomes (*c-e*). Furthermore in numerous instances, 5-nm gold particles are found over IF in deeper cytoplasmic regions relative to the 10-nm gold particles. Bars, 200 nm.

tron microscopic observations of negatively stained specimens (data not shown). Taken together, these data suggest that the inability of the desmosomal keratin to polymerize when present in the unfractionated urea-soluble desmosomal fraction is not due to some particular properties of these keratins, but to some interaction between these keratins and other protein(s) of the urea-soluble desmosomal fraction.

We have examined whether IFAP 300 binds to desmosomal keratin IF. For these experiments, we used buffer conditions under which keratin IF were assembled and pelleted at 100,000 *g*. Using the same buffer, IFAP 300 alone is recovered in the 100,000 *g* supernatant. However, when IFAP 300 is incubated with purified desmosomal keratin IF for 1–2 h at room temperature it is recovered along with the keratin IF in the 100,000 *g* pellet (Fig. 8). Since IFAP 300 alone is not pelletable under identical conditions, its presence in the keratin pellet appears to be due to its interaction with keratin IF. Furthermore, this does not appear to be due to nonspecific binding of IFAP 300, as we could not detect binding of IFAP 300 to myosin filaments (Fig. 8). Myosin was chosen as a control since, like keratin, it possesses an α -helical rod domain. In addition, previous results have demonstrated that IFAP 300 does not bind to F-actin *in vitro* (Lieska et al., 1985).

Are IFAP 300 and Plectin the Same Protein?

It has been proposed that plectin and IFAP 300 are very similar, perhaps even identical proteins (Herrmann and Wiche, 1987). To examine this question further, we have determined the reactivity of our antibodies with plectin from C6 glioma cells, where this protein was first identified. The anti-IFAP 300 monoclonal antibody exhibits no obvious cross reactivity with C6 glioma plectin (Fig. 9). Conversely, two plectin monoclonal antibodies (1D8 and 10F6; Foisner et al., 1991) react strongly with plectin from C6 glioma cells, but not with IFAP 300 from BHK cells. On the other hand, the polyclonal anti-IFAP 300 shows a reaction with both IFAP 300 and plectin. These results suggest that these two proteins have both common and unique epitopes.

There are also significant differences between two-dimensional tryptic maps of radiolabeled peptides derived from

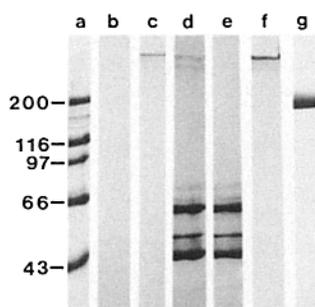


Figure 8. Coomassie blue-stained 7% SDS gel loaded with: (lane a) molecular mass markers (molecular mass is indicated in kilodaltons); (lane b) the 100,000 *g* pellet and (lane c) supernatant of IFAP 300 purified from BHK cells and dialyzed against keratin assembly buffer; note that under these conditions IFAP 300 is soluble; (lane d) the 100,000 *g* pellet obtained

from desmosomal keratin IF incubated with IFAP 300; under these conditions IFAP 300 is pelleted; (lane e) the 100,000 *g* pellet obtained from desmosomal keratin IF; note that it contains no high molecular mass species; (lane f) the 100,000 *g* pellet and (lane g) supernatant obtained from rabbit skeletal muscle myosin filaments incubated with IFAP 300: IFAP 300 does not cosediment with myosin filaments.

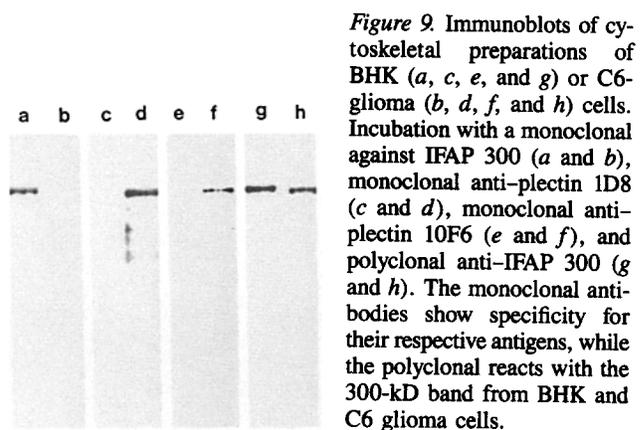


Figure 9. Immunoblots of cytoskeletal preparations of BHK (a, c, e, and g) or C6 glioma (b, d, f, and h) cells. Incubation with a monoclonal against IFAP 300 (a and b), monoclonal anti-plectin 1D8 (c and d), monoclonal anti-plectin 10F6 (e and f), and polyclonal anti-IFAP 300 (g and h). The monoclonal antibodies show specificity for their respective antigens, while the polyclonal reacts with the 300-kD band from BHK and C6 glioma cells.

125 I-IFAP 300 and 125 I-plectin. The differences include both the number of radiolabeled peptides as well as their migration pattern (Fig. 10). We have also started to compare IFAP 300 and plectin with respect to their primary sequence. A λ gt11 cDNA library prepared from BHK-21 cells has been screened with the monoclonal anti-IFAP 300 and a clone reacting with that antibody has been selected and subcloned. Mouse polyclonal antibodies directed against the fusion protein encoded by this clone have also been prepared. By immunoblotting these antibodies react with IFAP 300 in BHK cells and in bovine tongue desmosomes (data not shown). By indirect immunofluorescence these antibodies produce staining patterns similar to those obtained with the other IFAP 300 antibodies (see above) in BHK, PME, and 804G cells (data not shown). Sequencing of this cDNA clone reveals a 296-bp insert with a single uninterrupted open reading frame coding for 98 amino acids. Comparison of this sequence with that of plectin shows that it has 43% identity with a region of the rod domain of plectin (Wiche et al., 1991) (Fig. 11). However, to obtain this value, seven gaps have to be introduced in the partial sequence of IFAP 300. Without introducing these gaps the percentage of identity with plectin falls to 25% (Fig. 11). Therefore there are significant differences between IFAP 300 and plectin at the level of their primary sequence.

Discussion

Desmosomes and hemidesmosomes are plasma membrane-associated structures which are specialized in cell-cell and cell-matrix adhesion, respectively. The anchorage of IF to these junctional complexes is thought to be in large part responsible for the formation of a supracellular filamentous network (Jones et al., 1982) that contributes to the mechanical properties of epithelial tissues (Bonifas et al., 1991; Coulombe et al., 1991a,b; Vassar et al., 1991; Lane et al., 1992). Since desmosomes and hemidesmosomes differ in their protein composition, it is usually assumed that the attachment of IF to these junctions is mediated through different proteins (Schwarz et al., 1990; Jones and Green, 1991; Garrod, 1993). However, using both morphological and biochemical techniques, we have demonstrated here that IFAP 300 is a protein common to both desmosomes and hemidesmosomes. Furthermore, the ultrastructural localization of IFAP 300 as well as its ability to bind to keratin IF *in vitro*

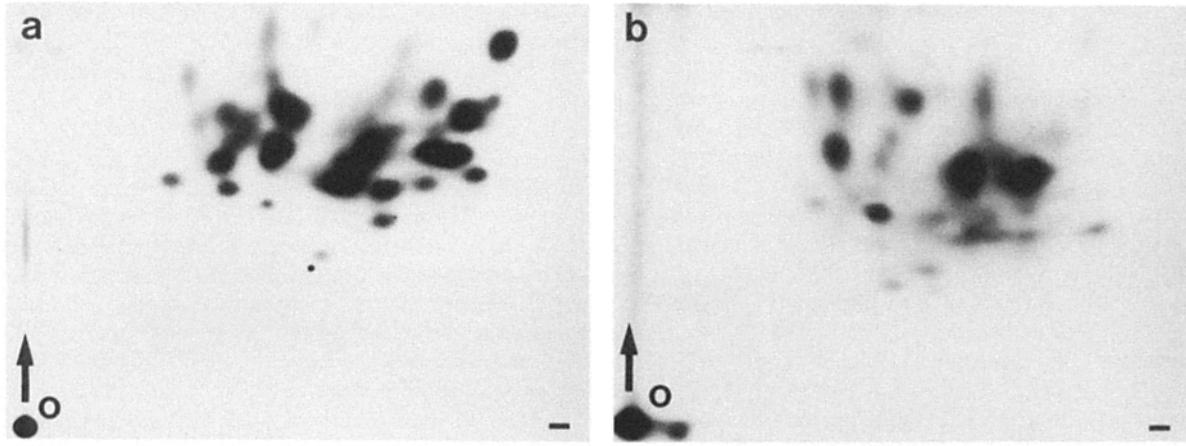


Figure 10. Two-dimensional maps of tryptic peptides derived from ^{125}I -IFAP 300 (*a*) and ^{125}I -plectin (*b*). Note the differences in the number of major iodinated peptides and in their migration profiles. "O," "-" and the arrow indicate, respectively, the origin, the cathode during electrophoresis, and the direction of ascending chromatography.

suggest that IFAP 300 is instrumental in connecting IF to desmosomes and hemidesmosomes.

This adds IFAP 300 to the list of proteins that have been proposed to contribute to the anchorage of IF to desmosomes and hemidesmosomes. For desmosomes this list includes: desmoplakin (Green et al., 1990; Stappenbeck and Green, 1992), desmoccalmin (Tsukita and Tsukita, 1985), B6P (Kapprell et al., 1988), and a 140-kD protein sharing antigenic determinants with lamin B (Cartaud et al., 1990). For hemidesmosomes the candidate linkers include: β -4 integrin (Quaranta and Jones, 1991), a 500-kD protein called HD1 (Hieda et al., 1992), and the 230-kD bullous pemphigoid antigen (BP 230) (Jones et al., 1986; Green et al., 1990). IFAP 300 is unique among all these proteins since it

is found both in desmosomes and hemidesmosomes as we show here by immunofluorescence on tissue and cells.

The ultrastructural localization of IFAP 300 indicates that this protein is concentrated at the point where IF are attached to the inner plaque of both desmosomes and hemidesmosomes. This is precisely the localization expected for a common linker molecule between IF and desmosomes or hemidesmosomes. For desmosomes, the other proteins that also fulfill this ultrastructural localization requirement are desmoplakin (Mueller and Franke, 1983; Jones and Goldman, 1985; Steinberg et al., 1986; Miller et al., 1987) and B6P (Kapprell et al., 1988). Desmoccalmin (Tsukita and Tsukita, 1985) and the 140-kD desmosomal protein (Cartaud et al., 1990) have been located in the desmosomal-dense plaque immediately subjacent to the plasma membrane, which does not support the idea that they function directly in linking IF to desmosomes. By immunogold electron microscopy HD1 (Hieda et al., 1992), BP 230 (Shimizu et al., 1985; Jones et al., 1986; Klatt et al., 1989; Owaribe et al., 1990; Tanaka et al., 1990), and β -4 integrin (Stepp et al., 1990; Jones et al., 1991) have been localized on the cytoplasmic side of the hemidesmosomal plaque, next to the site of anchorage of IF. This localization is consistent with a possible role of these proteins in connecting IF to hemidesmosomes.

In our immunogold labeling experiments of desmosomes, the quantitative analysis of the distribution of the gold particles shows an overlap between the distribution of IFAP 300 and desmoplakin. This raises the interesting possibility that desmoplakin and IFAP 300 interact with each other in desmosomes. Similarly, in hemidesmosomes, the overlap between the distribution of the gold particles labeling IFAP 300 and those labeling BP 230 may reflect binding between these two proteins. Obviously, extensive biochemical studies will have to be carried out to determine if IFAP 300 does indeed bind to desmoplakin in desmosomes and to BP 230 in hemidesmosomes. IFAP 300 is not only present at the site of insertion of IF into the inner plaque of desmosomes and hemidesmosomes, but also for a distance of about 100 nm over the IF inserting into the inner plaque. This suggests that IFAP 300 may also function in stabilizing or cross-linking

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1  QKLRKLEEKQA.....RKAQREAEAEAEERERKRLESQREAEWK...KEEE 42
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1184 QLERTLQEEHVTVTQLRREAEERRAQQAERAREEAERELERWQLKANE 1233
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
43  RLRLK.....EEQKEEERKAR.....EEQARREHE... 68
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1234 ALRLRLQAEVEVAQKSLAQADAERQKEEAERARRRGAEEQAVRQRELA 1283
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
69  ..EYKLEKEA.....FVVEEERG...SETMTEEQSHSFLT 98
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1284 EQELEKQRQLAEGTAQORLAAEQELIRLRAETEQGENERQLE 1326

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a

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1  QKLRKLEEKQARKAQREAEAEAEERERKRLESQREAEWKKEERLRLKKEEQ 51
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1196 VTVTQLRREAEERRAQQAERAREEAERELERWQLKANEALRLRLQAEVE 1244
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
52  EEEERKAREEQARREHEEYKLEKEAFVVEEERGSETMTEEQSHSFLT 98
   | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1245 AQKSLAQADAERQKEEAERARRRGAEEQAVRQRELAEQELEKQR 1291

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b

Figure 11. Best possible alignments of the partial sequence of IFAP 300 (*top*) with the sequence of plectin (*bottom*), with (*a*) or without (*b*) the introduction of gaps. Vertical bars between the two sequences denote identical amino acids and dots indicate conservative amino acid substitutions. This sequence data is available from EMBL/GenBank/DBJ under accession number X77631 (C. Griseus IFAP 300 mRNA).

IF before they anchor into desmosomes and hemidesmosomes.

In addition to being localized at the site of insertion of IF into desmosomes and hemidesmosomes, any protein directly involved in the linkage of IF to these adhesion sites must also bind to IF. Direct binding of a desmosomal or hemidesmosomal protein to polymerized keratin IF *in vitro* has been demonstrated only for desmocalmin, a 240-kD protein present in desmosomes but not in hemidesmosomes (Tsukita and Tsukita, 1985), and in this study, for IFAP 300. In addition, IFAP 300 has also been shown to bind to vimentin IF (Lieska et al., 1985). Therefore, it would be interesting to know if IFAP 300 is concentrated subjacent to desmosomes in cells in which vimentin IF are associated with plaque regions, such as in the cases of arachnoidal and dendritic reticulum cells (Franke and Moll, 1987; Achtstatter et al., 1989).

Uncertainties remain as to whether desmosomal and hemidesmosomal proteins other than IFAP 300 and desmocalmin bind directly to IF *in vitro*. Solid phase overlay assays show that B6P (Kapprell et al., 1988) and the 140-kD lamin B-like desmosomal protein (Cartaud et al., 1990) can associate with IF proteins. However, in the case of B6P, the physiological significance of the binding is unclear since the IF protein used for the assay (cytokeratin 18) is found only in simple epithelia, which do not express B6P (Kapprell et al., 1988). Biochemical evidence supporting the idea that desmoplakin may bind to IF is the finding that keratin IF cannot be assembled from a urea-soluble desmosomal fraction greatly enriched in both desmoplakin and keratin (Jones et al., 1988). This is likely to be the result of an interaction between keratin polypeptides and some other protein(s), perhaps including desmoplakin, present in the urea-soluble desmosomal fraction. Further evidence in support of this possibility is our present finding that once free of other urea-soluble desmosomal proteins (of which desmoplakin is the major constituent), the keratins isolated from desmosomes are polymerization competent. In addition, transient transfection experiments show that the COOH-terminal region of desmoplakin colocalizes with the endogenous IF network, and that this binding may result in the disruption of this network (Stappenbeck and Green, 1992). This latter result may be related to the finding that the COOH-terminal domain of desmoplakin contains repeated sequences of acidic and basic residues which are also present in segment 1B of the rod domain of IF proteins (Green et al., 1990). This region has been found to be important in the protein-protein interactions required for IF assembly. Similar repeats are also found in BP 230 (Stanley et al., 1988; Tanaka et al., 1991). It has been proposed that these repeats provide the basis for ionic interaction between IF and desmoplakin in desmosomes or between IF and BP 230 in hemidesmosomes (Green et al., 1990). However, direct binding of purified desmoplakin to keratin IF is not seen *in vitro* using cosedimentation assays (O'Keefe et al., 1989).

On the basis of all of the available data, IFAP 300 appears to be the best candidate characterized to date for linking IF to desmosomes and hemidesmosomes. In support of this contention, it is the only known protein to be present in both desmosomes and hemidesmosomes at the site of insertion of IF into the most distal regions of the submembranous plaque. It also binds keratin IF *in vitro*. Of course, this does not exclude other proteins from playing similar and/or syner-

gistic roles in the binding of IF to cell surface adhesion sites. For example, in the case of desmosomes, a complex between IFAP 300 and desmoplakin may serve in the attachment of IF, while in hemidesmosomes the same function may be served by a complex between IFAP 300 and BP 230.

It has been suggested that IFAP 300 is very similar to plectin, an IFAP of similar molecular weight (Herrmann and Wiche, 1987). However, there are a number of significant differences between the two proteins as demonstrated in this study. These include the lack of monoclonal antibody cross-reactivity, differences in two-dimensional tryptic maps, and preliminary data from the partial sequence of an IFAP 300 cDNA clone. However, sequence comparisons show that some residues are conserved between the 98-amino acid stretch of sequence derived from IFAP 300 and a region of the rod domain of plectin. Further sequencing is in progress in our laboratory, and should shed light on whether IFAP 300 and plectin are totally different proteins or are related proteins, or perhaps isoforms, belonging to the same family of proteins. Immunofluorescence experiments have suggested that plectin is also present in desmosomes and hemidesmosomes (Wiche et al., 1983, 1984). Immunoelectron microscopy using peroxidase-conjugated secondary antibodies also suggested that plectin is enriched at the level of the desmosomal plaque (Wiche et al., 1983). However, the immunoperoxidase technique does not allow for the precise localization of the antigen within the different domains of the desmosome as is the case with the immunogold technique. Furthermore, it has not been determined whether the staining seen with plectin antibodies reflects the actual presence of this protein within desmosomes and hemidesmosomes or a cross-reactivity with other desmosomal or hemidesmosomal proteins. This is an important issue since there are sequence similarities between plectin, BP 230, and desmoplakin (Green et al., 1990, 1992; Tanaka et al., 1991; Wiche et al., 1992).

In epithelial tissues, the localization of IFAP 300 is concentrated in regions closely associated with desmosomes and hemidesmosomes and does not extend over the entire keratin network. This distribution differs from that described in BHK-21 cells, where IFAP 300 is colocalized with the vimentin IF network throughout interphase (Yang et al., 1985) and mitosis (Skalli et al., 1992). Since IFAP 300 binds to keratin IF *in vitro*, the restricted localization of IFAP 300 at the cell surface of keratinocytes suggests that factors, such as phosphorylation or the binding of other associated proteins, may prevent the association of IFAP 300 with the overall keratin IF network; the exception being the points at which IF attach to the cell surface. The differential distribution of IFAP 300 in fibroblasts and in epithelial cells raises the possibility that IFAP 300 is a multifunctional protein, serving as an IF cross-linker in fibroblasts (Lieska et al., 1985; Yang et al., 1985) and as a connector of IF to desmosomes and hemidesmosomes in epithelial cells. Therefore, IFAP 300 may be important in determining the cell type-specific organization of IF networks, possibly through interactions with tissue-specific proteins, such as desmoplakin and BP 230 in epithelial cells. In turn, such interactions at the level of single cells probably translate into determining the tissue-specific functions of IF networks, including the formation of the supracellular cytoskeletal networks of keratin thought to be responsible for the stabilization of cell-cell

interactions and the mechanical properties of stratified squamous epithelia (Coulombe et al., 1991a).

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