Identification of trichoplein, a novel keratin filament-binding protein

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Accepted 29 November 2004
Journal of Cell Science 118, 1081-1090 Published by The Company of Biologists 2005
doi:10.1242/jcs.01667

Summary

Keratins 8 and 18 (K8/18) are major components of the intermediate filaments (IFs) of simple epithelia. We report here the identification of a novel protein termed trichoplein. This protein shows a low degree of sequence similarity to trichohyalin, plectin and myosin heavy chain, and is a K8/18-binding protein. Among interactions between trichoplein and various IF proteins that we tested using two-hybrid methods, trichoplein interacted significantly with K16 and K18, and to some extent with K5, K6a, K8 and K14. In in vitro co-sedimentation assays, trichoplein directly binds to K8/18, but not with vimentin, desmin, actin filaments or microtubules. An antibody raised against trichoplein specifically recognized a polypeptide with a relative molecular mass of 61 kDa in cell lysates. Trichoplein was immunoprecipitated using this antibody in a complex with K8/18 and immunostaining revealed that trichoplein colocalized with K8/18 filaments in HeLa cells. In polarized Caco-2 cells, trichoplein colocalized not only with K8/18 filaments in the apical region but also with desmoplakin, a constituent of desmosomes. In the absorptive cells of the small intestine, trichoplein colocalized with K8/18 filaments at the apical cortical region, and was also concentrated at desmosomes. Taken together, these results suggest that trichoplein is a keratin-binding protein that may be involved in the organization of the apical network of keratin filaments and desmosomes in simple epithelial cells.

Key words: Intermediate filament, Keratin, Plectin, Trichohyalin, Trichoplein

Introduction

Intermediate filaments (IFs) are major components of the cytoskeleton and nuclear envelope in most types of eukaryotic cells (Franke, 1987; Fuchs and Weber, 1994; Fuchs and Cleveland, 1998; Coulombe and Omary, 2002; Herrmann et al., 2003). Although structural components of other major cytoskeletal proteins, actin and tubulin, are highly conserved in different cell types, the constituent proteins of IFs show intriguing molecular diversities and are expressed in tissue-specific programs, which makes them ideal molecular markers for the differentiation state in developmental biology and pathology studies (Steinert and Roop, 1988). The keratin subfamily, which is preferentially expressed in epithelial cells, has over 20 members (keratin 1-keratin 20) that form obligate non-covalent heteropolymers of at least one type I (keratin 9-keratin 20) and one type II keratin (keratin 1-keratin 8) (Moll et al., 1982). In epithelial cells, keratin filaments are typically organized in a cytoplasmic reticular network of anastomosing filament bundles involving their noncovalent linkage at the surface of nucleus and at cell adhesion complexes. This adhesion machinery consists of desmosomes, mediating adhesion between cells, and hemidesmosomes, which mediate the adhesion of epithelial cells to the underlying basal lamina (Green and Gaudry, 2000; Leung et al., 2002; Getsios et al., 2004). A primary role that has been ascribed to various keratin filament networks of stratified squamous epithelia is to impart mechanical integrity to cells, without which, the cells become fragile and prone to rupture (Hutton et al., 1998). Disruption of the keratin IF network in epidermal keratinocytes via the targeted expression of dominant-negative keratin mutants (Coulombe et al., 1991; Vassar et al., 1991; Takahashi and Coulombe, 1996) or the introduction of null mutations (Lloyd et al., 1995) results in lysis of the targeted cell population whenever the skin of such mice is subjected to trivial mechanical trauma. Mutations in keratin genes, weakening the structural framework of cells, increase the risk of cell rupture and cause a variety of human skin disorders (Coulombe, 1993; Fuchs and Cleveland, 1998).

Keratin 8 and 18 (K8/18) are the major components of the IFs of simple or single-layered epithelia, as found in the gastrointestinal tract, liver, and exocrine pancreas, from which many carcinomas arise. Gene targeting techniques have been used to elucidate the function of K8/18. K8 knockout mice in one strain died around day 12 from undetermined tissue damage (Baribault et al., 1993), whereas in a different strain, they survived to adulthood but colorectal hyperplasia and inflammation were present (Baribault et al., 1994). K18-null mice were fertile and had a normal life span, but older K18-null mice developed a distinct liver pathology with abnormal hepatocytes containing K8-positive aggregates that resembled

the Mallory bodies seen in human livers in alcoholic hepatitis (Magin et al., 1998). Together with the report describing a mutation in the K18 gene in a patient with cryptogenic cirrhosis (Ku et al., 1997), K18 mutations may possibly cause or result in a predisposition to liver disease (Omary and Ku, 1997). To investigate the role of keratins in alcoholic hepatitis, the response of the K8 knockout mouse to 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) was investigated. Intoxicated K8 knockout mice suffer from extensive porphyria and progressive toxic liver damage without the formation of Mallory bodies (Zatloukal et al., 2000). This study also suggested that the relative ratio of K8 to K18 determines whether Mallory bodies are formed under certain conditions, with K8 playing a protective role in certain types of toxic liver injury (Zatloukal et al., 2000).

To address the molecular basis for the function of K8/18, we previously screened a yeast two-hybrid library using K18 as bait, and identified Mrj (Izawa et al., 2000) and TRADD (Inada et al., 2001) as K18-binding proteins. In the present work, to further dissect the function of K8/18, we screened a yeast two-hybrid library, again using K8 as bait. Among the positive clones, we identified a clone encoding the C-terminal portion of human trichoplein, a novel protein that shows a low degree of sequence similarity to trichohyalin, plectin and myosin heavy chain. We report here evidence for the in vivo association of trichoplein with K8/18 filaments.

Materials and Methods

Materials

Mouse ZO-1 monoclonal antibody (T8-754) was a gift from Sh. Tsukita (Kyoto University). Mouse β-catenin monoclonal antibody (BD Transduction Laboratories, San Diego, CA), mouse desmoplakin 1&2 monoclonal antibody (Progen, Heidelberg, Germany), guinea pig keratin 8/18 polyclonal antibodies (Progen), mouse K18 monoclonal antibody (CY-90; Sigma, St Louis, MO), mouse K8 monoclonal antibody (Progen), mouse tubulin monoclonal antibody (Sigma), mouse desmin monoclonal (DakoCytomation, Japan) antibody, rat PECAM monoclonal antibody (BD Pharmingen, San Diego, CA), Alexa 488-labeled antibody (Molecular Probes, Eugene, OR), FluoroLink Cy3-linked antibody (Amersham Pharmacia Biotech, UK), and rhodamine-conjugated phalloidin (Molecular Probes) were purchased. Mouse neurofilament (NF) monoclonal antibody has been characterized (Inagaki et al., 1996).

Yeast two-hybrid screening

pGEX-2TH-K18 and -K8 plasmids were kindly provided by H. Eto (Massachusetts General Hospital, Boston, MA). pET8c-K5 and -K14 plasmids were kindly provided by E. Fuchs (The University of Chicago, Chicago, IL). Yeast vectors harboring neurofilament (NF)-L, -M, and -H were general gifts from R. Liem and C. Leung (Columbia University College of Physicians and Surgeons, New York, NY). DNA encoding full-length K8 was cloned into the yeast GAL4 DNA-binding domain vector pYTH9α. The resulting plasmid, pYTH9α-K8 was used in the two-hybrid screen of a human liver cDNA library fused to pACT2 vector (BD Biosciences Clontech, Japan), following the Matchmaker Two-hybrid System protocol (BD Biosciences Clontech). Positive clones were screened for their potential to grow on selective medium containing 25 mM 3-aminotriazole and for the expression of β-galactosidase. Yeast DNA was recovered and transformed into *Escherichia coli*. Plasmids containing cDNA clones were identified by restriction mapping and further characterized by DNA sequencing. Subsequent two-hybrid interaction analyses were carried out by co-transformation of plasmids containing the GAL4 DNA-binding (pGBD) and -activation (pGAD) domains into *Saccharomyces cerevisiae* strain Y190.

Cloning of full-length trichoplein and DNA constructs

Standard DNA protocols were used (Sambrook et al., 1989). The full-length cDNA of human trichoplein was amplified using human liver Marathon-Ready cDNA (BD Biosciences Clontech) and PyroBest polymerase (Takara, Japan) with a set of primers designed according to the cDNA sequence deposited in GenBank (accession number BC004285). Sequences of these constructs were verified by DNA sequencing.

Purification of recombinant proteins

Recombinant trichoplein was expressed in *E. coli* as a maltose-binding protein (MBP)-tagged protein, using pMal vector (Qiagen, Japan). Expression and purification of the MBP-tagged trichoplein were done according to the manufacturer's protocol (Qiagen). The C-terminal region of trichoplein was expressed as glutathione S-transferase (GST) fusion protein in *E. coli* and purified on glutathione-agarose beads, essentially as described (Smith and Johnson, 1988). The expression and purification of His_6-tagged trichoplein were performed according to the manufacturer's protocol (Qiagen). Recombinant human K8, K18, desmin and mouse vimentin expressed in *E. coli* were prepared as described (Izawa et al., 2000). GST pull-down assay was carried out as described (Izawa et al., 2002)

IF assembly and co-sedimentation assay

In vitro IF assembly and co-sedimentation assay was also carried out as described (Izawa et al., 2000). Polymerized IF proteins, heterotypic complexes of K8/18, vimentin and desmin, were incubated with or without MBP or MBP-trichoplein. The assembled complexes were subjected to centrifugation at 12,000 ***g*** for 30 minutes followed by SDS-PAGE analyses.

Preparation of tubulin and microtubule-binding analyses

Pure tubulin was prepared from the bovine brain as described (Murata et al., 1992). The amount of trichoplein bound to microtubules was determined by co-sedimentation assay as described (Itoh et al., 1997). Briefly, trichoplein (0.25 μM) and polymerized tubulin (5 μM) were incubated in 0.1 M PIPES buffer (pH 6.9) containing 0.5 mM $MgSO_4$ and 1 mM EGTA. After a 10-minute incubation at 37°C, the samples were centrifuged at 100,000 ***g*** for 30 minutes at 37°C, then, aliquots of the supernatants and pellets were examined using SDS-PAGE.

Actin co-sedimentation assay

The amount of trichoplein bound to polymerized actin was determined in a co-sedimentation assay. Trichoplein (2.5 μM) and polymerized actin (3.5 mg/ml) were incubated in 20 mM Tris-HCl (pH 8.0) containing 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 mM ATP. After a 60-minute incubation at 25°C, the samples were centrifuged at 250,000 ***g*** for 10 minutes in a TLN-100 ultracentrifuge (Beckman Coulter, Japan). Aliquots of the supernatants and pellets were examined by SDS-PAGE.

Cell culture

HeLa cells and human bladder cell carcinoma T24 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin in an 95% air/5% CO_2 atmosphere with constant humidity. DJM-1 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 20 ng/ml

EGF, 0.4 μg/ml hydrocortisone, and 84 ng/ml cholera toxin. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.01 mg/ml human transferrin.

Transfection

cDNA encoding full-length trichoplein was cloned into the mammalian expression vector pEGFP-C1 (BD Biosciences Clontech) for expression of GFP-tagged proteins. HeLa cells were seeded at a density of 2×10^4 cells/ml onto 13-mm glass coverslips in six-well dishes the day before lipofection with 1 μg of each plasmid, using Lipofectamine (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocols. 16 hours after lipofection, the cells were fixed for immunofluorescence studies.

Generation of trichoplein antibody and immunoblotting

Trichoplein antibody was produced in rabbits injected with recombinant His_6-tagged trichoplein protein, and affinity-purified with recombinant GST-trichoplein fusion protein. HeLa, T24, and DJM-1 cells were lysed in SDS sample buffer, sonicated and boiled. Whole lysates of approximately 2×10^4 cells were then loaded in the lanes, resolved by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Atto, Japan). The blots were incubated with trichoplein antibody and horseradish-peroxidase-conjugated secondary antibody, and immunoreactive bands were visualized using chemiluminescence detection reagents (NEN Life Sciences, Boston, MA). To confirm specificity of the trichoplein antibody, it was preabsorbed by GST-trichoplein fusion protein, as a control. Lysates of proteins of various tissues from rats were also subjected to immunoblotting with the trichoplein antibody.

Immunoprecipitation

Cells were lysed on ice for 20 minutes in lysis buffer consisting of 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10 μM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin. Lysates were clarified by centrifugation at 12,000 ***g*** for 30 minutes. Endogenous trichoplein was immunoprecipitated from cell lysates with trichoplein antibody or control rabbit IgG, and protein A agarose beads, and washed four times with lysis buffer. Immunoprecipitates were analyzed by immunoblotting with primary antibodies and horseradish-peroxidase-conjugated secondary antibodies.

Immunofluorescence

HeLa cells grown on 13-mm coverslips were fixed by incubation for 10 minutes in 3.7% formaldehyde in PBS and then were permeabilized with 0.2% Triton X-100 for 10 minutes. For double immunostaining with trichoplein antibody and K18 monoclonal antibody (CY-90), cells were incubated with trichoplein antibody followed by Alexa 488-labeled rabbit antibody (Molecular Probes). Next the cells were incubated with K18 antibody followed by FluoroLink Cy3-linked mouse antibody (Amersham Pharmacia Biotech). To visualize actin filaments, cells were reacted with rhodamine-conjugated phalloidin (Molecular Probes). To visualize microtubules, cells were incubated with tubulin monoclonal antibody (Sigma), followed by FluoroLink Cy3-linked mouse antibody. A Radiance confocal microscope system (BioRad, Hercules, CA) was used to examine the coverslips.

Electron microscopic immunocytochemistry

HeLa cells cultured on coverslips were exposed to a fixative consisting of 2% glutaraldehyde and 2% formaldehyde in 0.85 M cacodylate buffer adjusted to pH 7.4. The fixed samples were then dehydrated in an ascending series of ethanol up to 99.5%, and then embedded in Lowicryl K4M resin (Polysciences, Warrington, PA) according to the manufacturer's protocol. Immunolabeling was carried out as described (Kachi et al., 1999).

Immunohistochemistry

Mouse (liver, brain, skeletal muscle, blood vessels of skeletal muscle, and hair follicle) and rat (small intestine) tissues were excised and then frozen in liquid nitrogen. These sections ~6 μm thick were then cut on a cryostat, mounted on glass slides, air-dried and fixed in 95% ethanol at 4°C for 30 minutes followed by 100% acetone at room temperature for 1 minute. Next they were rinsed in PBS, blocked with 1% BSA/PBS for 15 minutes, and incubated with primary antibodies. After three washes in PBS, samples were incubated for 30 minutes with secondary antibodies.

Results

Identification of trichoplein as a keratin-interacting protein

To identify proteins that bind keratin IFs, we screened a human liver cDNA library, using the yeast two-hybrid technique and full-length K8 as bait. Among the positive clones, we obtained a clone that encoded the C-terminal region (amino acid residues 73-498) of a human novel protein, which is deposited in

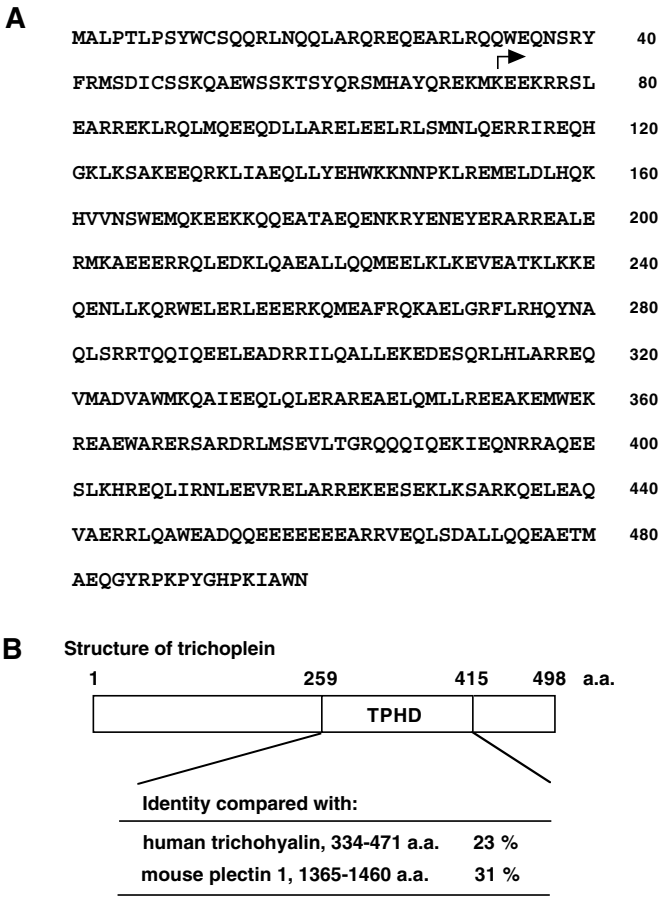


Fig. 1. Identification of trichoplein as a keratin-interacting protein. (A) Amino acid sequence of trichoplein from the full-length cDNA. The sequence contained in the original yeast clone is indicated with an arrow. (B) Scheme of the trichoplein protein with the trichohyalin/plectin homology domain (TPHD). Numbers refer to amino acid position.

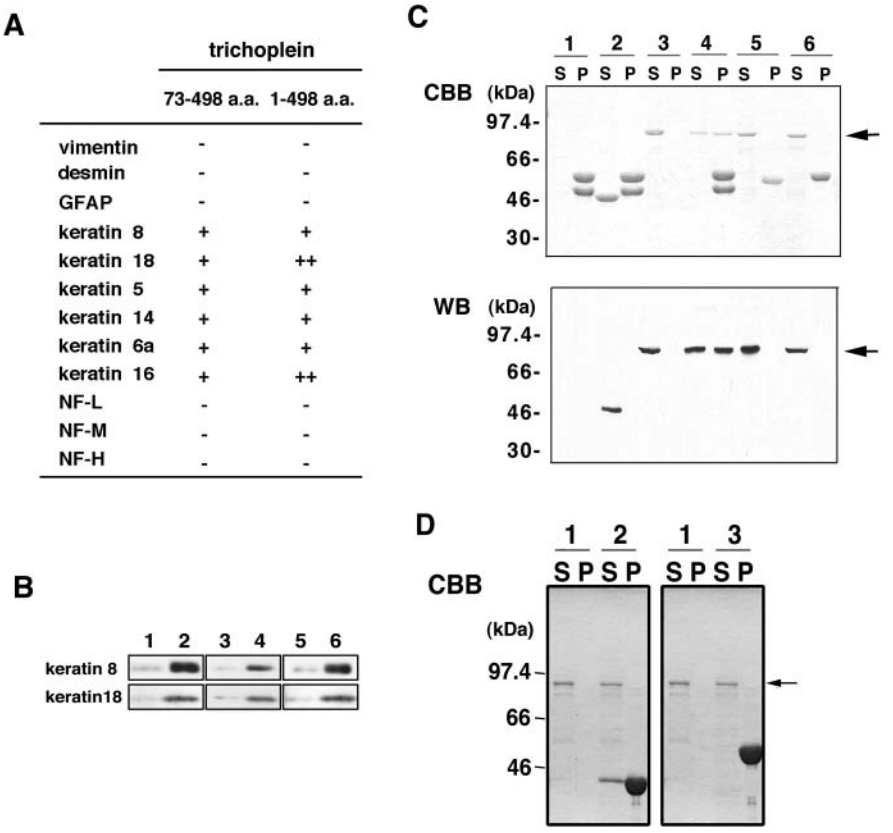
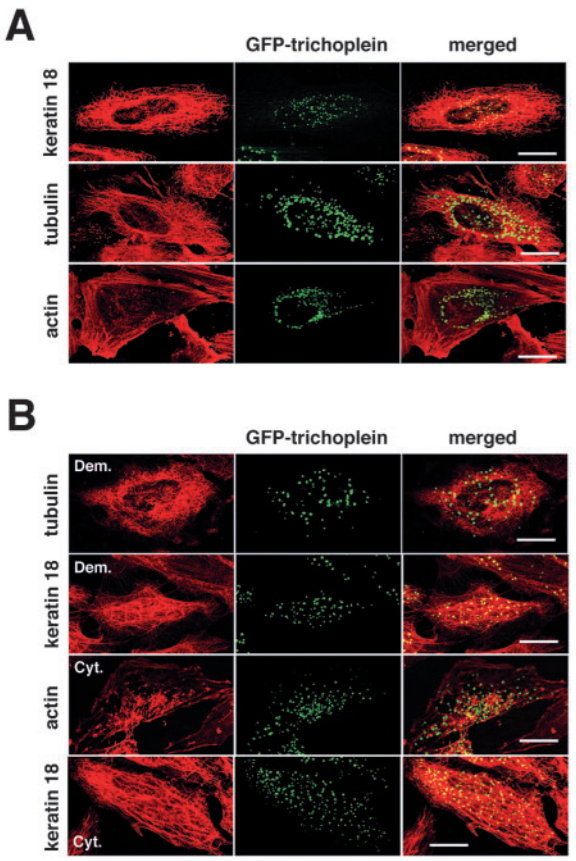


Fig. 2. In vitro interaction of trichoplein with keratin IFs. (A) Interactions of the C-terminal region of trichoplein (73-498 amino acids) or full-length trichoplein (1-498) with various IFs in the two-hybrid system. Y190 cells co-transformed with various pGBD-IFs and the pGAD-C-terminal region of trichoplein or pGAD-full-length trichoplein were selected in tryptophan- and leucine-free media and subjected to a β-galactosidase filter assay. The numbers of plus signs represent the relative rates that the transformed yeast colonies turned blue after incubation at 30°C on filters: +++, <3 hours; ++, 3-8 hours; +, >8 hours. Minus signs represent colonies remaining white at 24 hours. Yeast co-transformed with pGBD-K8 and pGAD-K18, which started to turn blue in less than 3 hours (designated +++) on the filter assay, served as a positive control. (B) Interaction of endogenous K8/18 with GST-trichoplein (73-498 a.a.). The extracts of HeLa (lanes 1 and 2), T24 (lanes 3 and 4) and DJM-1 (lanes 5 and 6) cells were incubated with either GST (lanes 1, 3, and 5) or GST-trichoplein (lanes 2, 4, and 6) fixed on glutathione beads and proteins attached to the beads were detected using K8 or K18 antibody. (C) Co-sedimentation analyses of trichoplein with K8/18. K8/18 filaments were assembled on their own (lane 1), or in the presence of MBP (lane 2) or MBP-trichoplein (lane 4). As a control, only MBP-trichoplein (lane 3) was incubated in the assembly buffer. Vimentin (lane 5) and desmin (lane 6) filaments were assembled in the presence of MBP-trichoplein. Samples of supernatant (S) and pellet (P) fractions were separated by SDS-PAGE, and then stained with Coomassie Brilliant Blue (CBB) (top panel), or detected with MBP antibody by immunoblotting (WB, bottom panel). Molecular weight markers are shown on the left. The arrows indicate the position of MBP-trichoplein. (D) Co-sedimentation of trichoplein with polymerized tubulin and actin. MBP-trichoplein was incubated with (lane 2) or without (lane 1) polymerized actin (left panel) or polymerized tubulin (right panel). Samples of supernatant (S) and pellet (P) fractions were separated on SDS-PAGE, and stained with CBB. The arrow indicates the position of MBP-trichoplein.

GenBank (accession numbers BC004285, AK092736 and AY007230). The open reading frame of the gene encodes a protein of 498 amino acids, which has a theoretical molecular mass of 61 kDa and an isoelectric point at 6.17 (Fig. 1A). A BLAST search with the whole protein sequence suggested a low degree of sequence similarity between the protein and trichohyalin, plectin and myosin heavy chain, and we termed this novel protein trichoplein. The region of amino acid residues 259-415 of human trichoplein, designated the trichohyalin/plectin homology domain (TPHD), showed 23% or 31% amino acid identity with the region of amino acid residues 334-471 of human trichohyalin or amino acid residues 1365-1460 of mouse plectin 1, respectively (Fig. 1B). As trichohyalin was reported to bundle keratin IFs into tight parallel arrays in a subset of hair follicles and tongue epithelial cells (Rothnagel et al., 1986; Fietz et al., 1993; Coulombe et al., 2000; Steinert et al., 2004) and plectin is one of founding members of plakin family proteins that attach cytoplasmic IFs to F-actin, microtubules and/or adhesion complexes (Wiche, 1998; Leung et al., 2002), we further examined the interaction of trichoplein with keratin IFs.

Direct association of trichoplein with keratin filaments in vitro

The full-length cDNA of trichoplein was amplified by PCR, using a set of primers designed according to the sequence in GenBank (BC004285). To confirm that full-length trichoplein interacts with K8 and to determine if trichoplein can bind to other IF proteins, we examined the interaction of full-length or C-terminal region (amino acid residues 73-498) of trichoplein with K5, K6a, K8, K14, K16, K18, vimentin, glial fibrillary acidic protein (GFAP), desmin, and neurofilament (NF) proteins in the two-hybrid system (Fig. 2A). The C-terminal region of trichoplein (the original two-hybrid fragment) interacted with all keratin proteins tested, but did not interact with Type III IFs or neurofilaments. In the same fashion, full-length trichoplein specifically interacted with keratin IFs, most strongly with K16 and K18 (Fig. 2A). We next checked the binding of endogenous K18 to an immobilized GST-trichoplein fusion protein (Fig. 2B). In this GST pull-down experiment, we found that GST-trichoplein could precipitate endogenous K18 from 1% Triton X-100-soluble lysates of HeLa, T24 and DJM-1 cells. Endogenous K8 was also precipitated by GST-trichoplein, suggesting that GST-trichoplein can bind to K8/18 complexes. To confirm the direct association of trichoplein with K8/18 filaments, we did a co-sedimentation assay (Fig. 2C,D). Incubation in filament assembly buffer induced a rapid polymerization of K8 and K18 (Fig. 2C, lane 1). As a control, recombinant MBP was not sedimented in the presence of K8/K18 (Fig. 2C, lane 2). In the absence of K8/18, MBP-



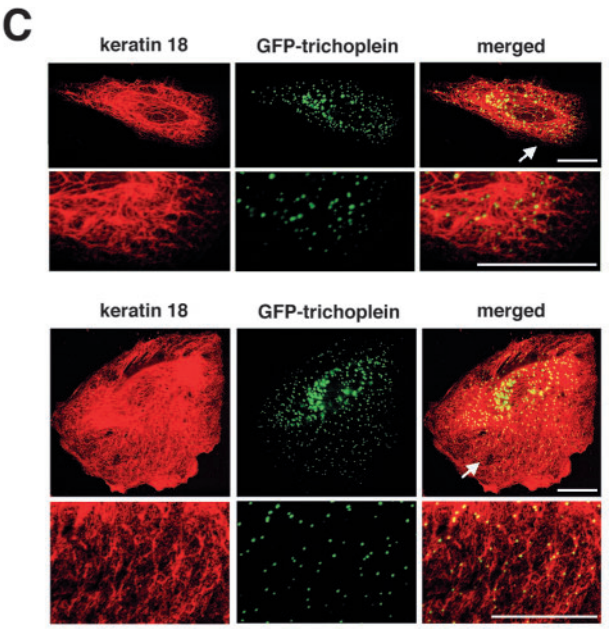


Fig. 3. Overexpressed GFP-tagged trichoplein is associated with K8/18 filaments in HeLa cells. (A) pEGFP-trichoplein vector was transfected into HeLa cells. The transfected cells were fixed 16 hours later and stained with K18 antibody, tubulin antibody or rhodamine-conjugated phalloidin. The merged images are also shown. (B) HeLa cells expressing GFP-trichoplein were treated with 10 ng/ml demecolcine (Dem.) for 100 minutes, or 5 μM cytochalasin B (Cyt.) for 15 minutes at 37°C. After the treatment, cells were fixed and stained with K18 antibody, tubulin antibody or rhodamine-conjugated phalloidin. (C) In some cells, GFP-trichoplein existed not only around nuclei but also in the cell periphery, where GFP-trichoplein markedly colocalized with K18. Higher magnification images (lower panels) of the areas indicated by arrows and merged images are also shown. Bar, 10 μm.

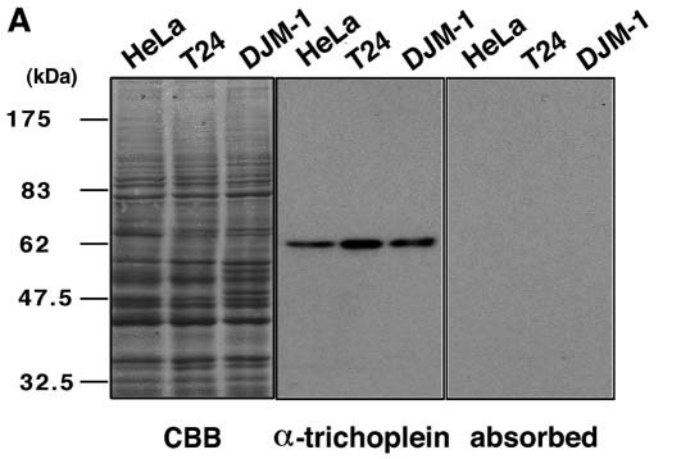
trichoplein was not sedimented (Fig. 2C, lane 3), but in the presence of K8/18, a substantial portion of MBP-trichoplein was precipitated with K8/18 (Fig. 2C, lane 4). In addition, trichoplein did not sediment with vimentin (Fig. 2C, lane 5) or desmin (Fig. 2C, lane 6), consistent with the results using the two-hybrid method. We further checked whether trichoplein could directly interact with actin filaments and microtubules (Fig. 2D). In the co-sedimentation assay, we detected no interaction of trichoplein with polymerized actin and tubulin. These results indicate that trichoplein interacts specifically with keratin IFs of all the major cytoskeletal proteins, in vitro.

Association of overexpressed GFP-tagged trichoplein with K8/18 filaments in HeLa cells

To check the association of trichoplein with K8/18 filaments in cells, we transiently expressed GFP-tagged full-length trichoplein (GFP-trichoplein) in HeLa cells, and observed the localization of the expressed trichoplein proteins (Fig. 3). The expressed GFP-trichoplein showed a punctate distribution around nuclei, which colocalized with K8/18 filaments, but not with microtubules or actin filaments (Fig. 3A). To show more clearly that trichoplein specifically interacts with K8/18 filaments and that this interaction is independent of tubulin and actin filaments, we treated HeLa cells expressing GFP-trichoplein with drugs that destroy tubulin or actin filaments, and then observed the colocalization of GFP-trichoplein and these filaments (Fig. 3B). The treatment of cells with demecolcine disrupted tubulin, but the localization of GFP-trichoplein did not markedly change compared to that of untreated cells, and GFP-trichoplein was still associated with K8/18 filaments. After the disruption of actin, the colocalization of GFP-trichoplein and K8/18 filaments was also similar to that of untreated cells. In some cells, the overexpressed GFP-trichoplein existed not only around nuclei but also in the cell periphery, where GFP-trichoplein markedly colocalized with K8/18 filaments (Fig. 3C). We also observed the subcellular localization of overexpressed myc-tagged trichoplein in HeLa cells, and found that the localization of myc-tagged trichoplein was almost similar to that of GFP-trichoplein (data not shown). These results suggest that overexpressed trichoplein is associated specifically with K8/18 filaments in HeLa cells. The localization of overexpressed trichoplein appears to be consistent with that of the endogenous trichoplein (Fig. 5A).

Interaction of trichoplein with keratin filaments in vivo

To explore the interaction between trichoplein and keratin IFs further, polyclonal antibodies raised against trichoplein were generated using purified His_6-tagged trichoplein (amino acid residues 277-488) as the immunogen. Affinity-purified trichoplein antibody specifically recognized a polypeptide with a relative molecular mass of approximately 61 kDa in HeLa, T24, and DJM-1 cell lysates (Fig. 4A). Preincubation of the antibody with GST-trichoplein fusion protein selectively inhibited the immunoreactivity (Fig. 4A). Using this antibody,



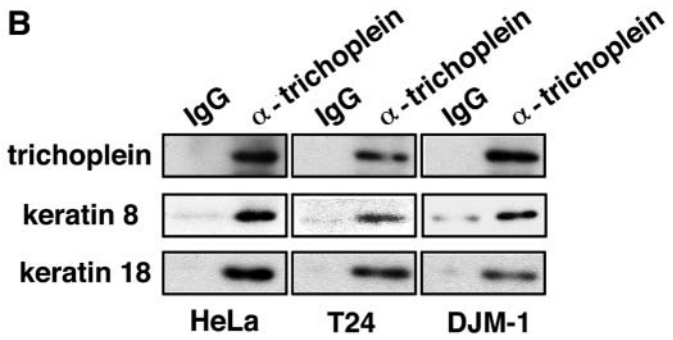


Fig. 4. In vivo association of trichoplein with K8/18. (A) Detection of trichoplein in HeLa, T24 and DJM-1 cells. Lysates from HeLa, T24 and DJM-1 cells were stained with CBB, detected with trichoplein antibody by immunoblotting (α-trichoplein), or immunostained with trichoplein antibody preabsorbed by GST-trichoplein protein (absorbed). Trichoplein antibody specifically recognized bands of about 61 kDa in samples. Molecular size markers are shown on the left. (B) Interaction of trichoplein with K8/18 in vivo. Immunoprecipitates were prepared from lysates of HeLa, T24 and DJM-1 cells using control rabbit IgG and trichoplein antibody. Each precipitate was subjected to immunoblot analysis with trichoplein, K8 or K18 antibody.

we precipitated complexes containing trichoplein proteins from the 1% Triton X-100-soluble fraction of HeLa, T24 and DJM-1 cell lysates (Fig. 4B). Trichoplein was immunoprecipitated with trichoplein antibody, but not by control rabbit IgG. K18 was co-immunoprecipitated with trichoplein using trichoplein antibody in these three cell types. A near-equivalent amount of K8 was also detected in the trichoplein immune complex. These results indicate that endogenous trichoplein is associated with K8/18 complexes in the Triton X-100-soluble fraction. To further characterize the trichoplein-K8/18 association, we investigated whether endogenous trichoplein could be recovered with salt- and detergent-resistant keratin preparations. For this, confluent HeLa cells were extracted with Triton X-100 and high salt buffers, and cytoskeletal proteins enriched in IFs were obtained as described (Franke et al., 1978; Franke et al., 1981). In the fractions, we could detect K8/18, but not trichoplein (data not shown). These results indicate that trichoplein is not very tightly associated with K8/18 filaments.

Trichoplein colocalizes with K8/18 filaments in HeLa cells

To determine the intracellular distribution of trichoplein and to determine if trichoplein colocalizes with K8/18 filament networks, HeLa cells were double-stained with trichoplein antibody and K18 antibody (Fig. 5A). Trichoplein was diffuse in the cytoplasm, and showed some punctate patterns. There was a significant overlap between trichoplein and K18, where trichoplein was remarkably associated with K8/18 filament bundles in the cell periphery (Fig. 5A). These associations of trichoplein with K8/18 filaments were also observed in other epithelial cell lines such as T24, MDCK and DJM-1 cells (data not shown). To evaluate the colocalization more precisely, we did an immunolabeling and an immunoelectron microscopy analysis (Fig. 5B). Clearly, trichoplein was present in close

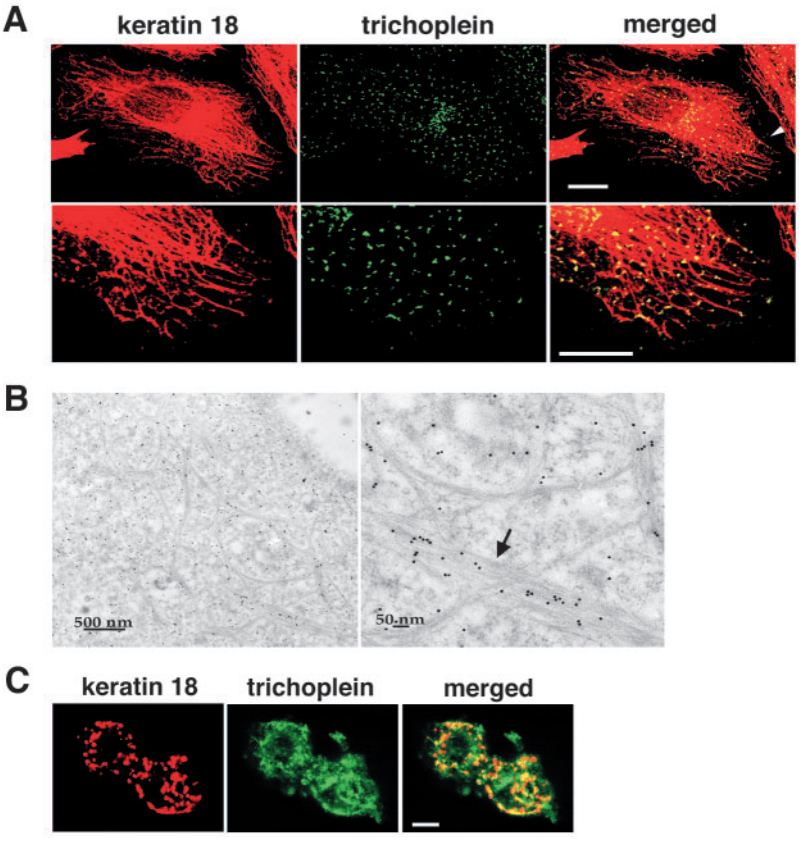


Fig. 5. Colocalization of trichoplein and K8/18 filaments in HeLa cells. (A) HeLa cells were double-stained with trichoplein and K18 antibodies. The enlargement of the area indicated by an arrowhead and merged images are also shown. (B) Electron microscopic immunolocalization of trichoplein in HeLa cells. Antibody binding sites were detected predominantly in keratin-rich regions (left). At high magnification (right), staining is obvious on the bundle of keratin filaments (arrow). (C) HeLa cells in mitosis were double-stained for K18 and trichoplein. Bar, 10 μm (A,C); 500 nm (B, left); 50 nm (B, right).

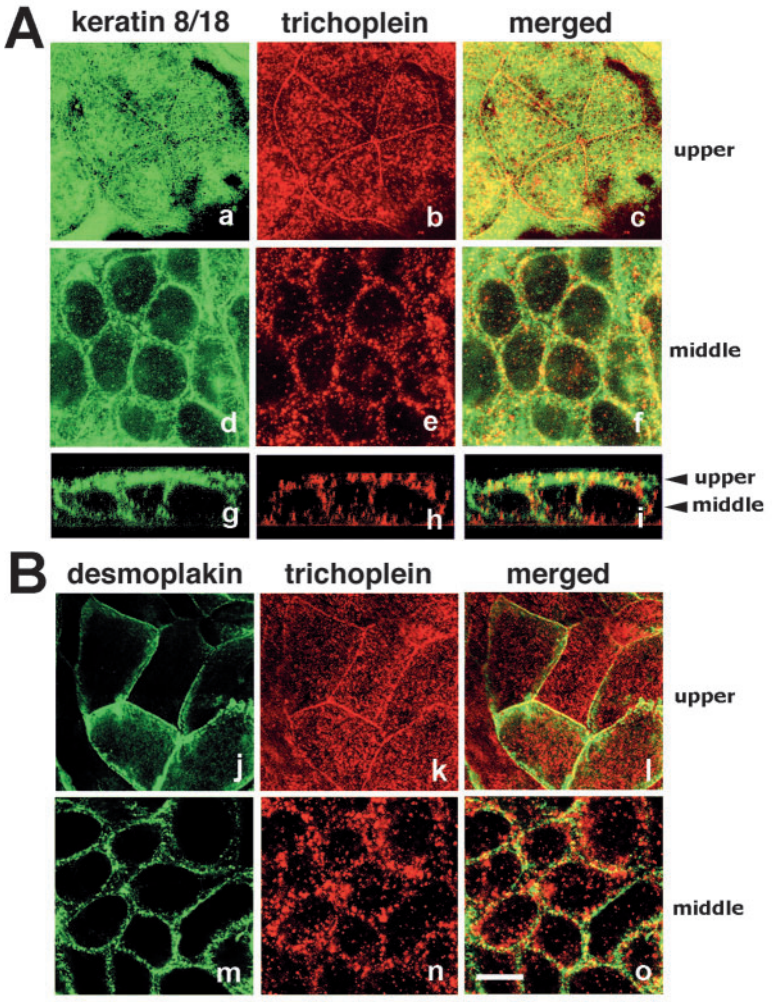


Fig. 6. Colocalization of trichoplein with K8/18 and desmoplakin in polarized Caco-2 cells. Caco-2 cells were cultured on laminin-coated cell culture inserts for more than 3 days. They were fixed and immunostained with antibodies as indicated and analyzed by laser-scanning confocal microscopy. Images a-f and j-o are in the *X-Y* plane. Images g-i are in the *Z*-section. (A) Caco-2 cells showed concentrations of K8/18 in the cortical cytoskeleton with higher density in apical region (a,d,g). At the upper and the middle level (a-i), trichoplein associates with K8/18 in a speckled pattern. Trichoplein also concentrated at the cell-cell border of the upper level (b). (B) Trichoplein colocalized with desmoplakin clearly at the cell-cell border of the upper level (j-l) and partially at the middle level (m-o). Bar, 10 μm.

vicinity with K8/18 filament bundles in HeLa cells. The IF organization of cultured cells is known to change dramatically in some situations (Franke et al., 1982; Werner et al., 2004). In HeLa cells, the keratin granules are seen during mitosis (Franke et al., 1982). We next checked if trichoplein colocalized with the keratin granules in HeLa cells (Fig. 5C). During mitosis, punctate K18 staining was observed as described (Franke et al., 1982), and trichoplein partially colocalized with the keratin granules (Fig. 5C). These results indicate that in HeLa cells, trichoplein is associated with K8/18 filaments both in interphase and in mitosis.

Colocalization of trichoplein with K8/18 and desmoplakin in polarized Caco-2 cells

To see whether trichoplein colocalizes with K8/18 in polarized simple epithelial cells, we used a human colon carcinoma cell line Caco-2, an extensively studied model of epithelial polarization that differentiates well in cell culture (Fig. 6). We confirmed the expression of trichoplein in Caco-2 cells by an immunoblot analysis (data not shown). In polarized Caco-2 cells, K8/18 signal was present in the apical cortical region, and extended throughout the lateral cortical region (Fig. 6A), as previously described (Salas, 1999). These authors suggested that a thin layer (a terminal web-like structure) of keratin IFs located beneath the apical membrane of a variety of simple epithelial cells participates in the organization of the apical pole in epithelial cells (Salas et al., 1997). Trichoplein was present in the apical cortical region as well as along the lateral membrane, and colocalized with K8/18 markedly in the apical region (Fig. 6A). In addition, trichoplein colocalized with desmoplakin at the cell-cell border of the upper level (Fig. 6B). These results indicate that in polarized Caco-2 cells, trichoplein colocalizes with K8/18 and desmoplakin, mainly in the cortical area.

Expression and distribution of trichoplein in various tissues

The expression and distribution of trichoplein in various tissues were then examined (Figs 7, 8). Immunoblotting analysis revealed that trichoplein was expressed ubiquitously (Fig. 7A). Frozen sections of liver were double-stained with K8/18 antibody and trichoplein antibody. In hepatocytes, trichoplein was exclusively concentrated and merged with K8/18 filaments in tubular structures, probably bile canaliculi (Fig. 7B). In brain or skeletal muscle, where keratin IFs were not expressed, trichoplein was not concentrated at neurofilament (NF)- or desmin-positive IFs (Fig. 7B). The staining of the hair follicle showed that trichoplein localized at the outer root sheath where K6/16 localized (Fig. 7C) and we found that blood vessels were also trichoplein-positive (Fig. 7B,D). These results indicate that trichoplein is expressed not only in epithelial tissues but also in non-epithelial tissues where keratin IFs are absent.

In the enterocytes of the small intestine, the K8/18 signal was typically concentrated in the apical region (terminal web) (Fig. 8A,C), as previously reported (Ameen et al., 2001). Trichoplein was markedly concentrated in the apical region, and was associated with K8/18 (Fig. 8A,C). In the apical region, trichoplein was also concentrated at cell-cell border and colocalized with desmoplakin (Fig. 8B,C). In vertical sectional views of the epithelial cells doubly stained with trichoplein antibody and antibodies to the junctional complex markers (ZO-1, β-catenin and desmoplakin 1&2) (Fig. 8C), trichoplein clearly overlapped with desmoplakins, but not with ZO-1 or β-catenin. These observations in the absorptive cells of small intestine are consistent with those of Caco-2 (Fig. 6).

Discussion

A major function shared by several types of cytoplasmic IFs is

to stabilize cellular architecture against the mechanical forces it is subjected to (Coulombe et al., 2000). IFs function together with a number of IF-associated proteins, which form cross-bridges between IFs and other cytoskeletal elements, integral membrane proteins and motor proteins (Houseweart and Cleveland, 1998; Herrmann and Aebi, 2000; Green and Gaudry, 2000; Leung et al., 2002; Getsios et al., 2004). Here we identified trichoplein, a novel protein, as a keratin-binding protein, using a yeast two-hybrid system, biochemical analysis and cell biological techniques. The open reading frame of the human trichoplein gene encodes a protein of 498 amino acid residues containing the domain that shows homology to trichohyalin and plectin (Fig. 1). In the two-hybrid assay, trichoplein interacted with keratin IFs, most strongly with K16 and K18, but not with other IF proteins including vimentin, GFAP, desmin, or neurofilaments. We showed that trichoplein is associated in vitro with K8/18 filaments via direct binding in a co-sedimentation assay (Fig. 2). Trichoplein associated in vivo with K8/18 complexes, and colocalized with K8/18 filaments (Figs 3-5). In polarized Caco-2 cells, trichoplein colocalized with K8/18 mainly in the apical submembrane domain and with desmoplakin in the apical junctional domain (Fig. 6). Trichoplein was expressed ubiquitously (Fig. 7A), and was present not only in epithelial tissues but also in non-epithelial tissues which lack keratin IFs (Fig. 7B,D). In the absorptive cells of small intestine, trichoplein colocalized with K8/18 filaments at the apical cortical region, and was also concentrated at desmosomes in the apical junctional domain (Fig. 8). Taken together, these results suggest that trichoplein binds to K8/18 filaments, in vivo, and may be a constituent of desmosomes.

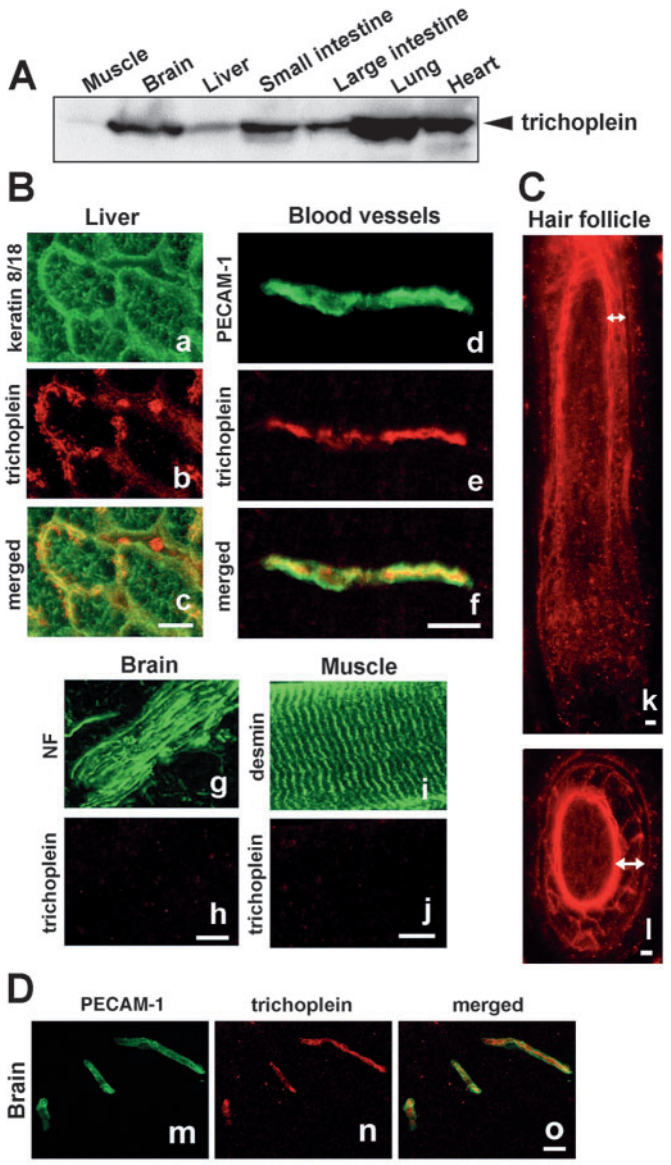


Fig. 7. Expression and distribution of trichoplein in various tissues. (A) Homogenates of various tissues from rats were immunoblotted with trichoplein antibody. Each lane contained 30 μg of protein from selected tissues. Trichoplein seemed to be expressed ubiquitously. (B) Double-staining of various tissues with trichoplein antibody and markers. In liver (a-c), trichoplein was exclusively concentrated and merged with K8/18 filaments in tubular structures (bile canaliculi). On the other hand, in brain (g,h) or skeletal muscle (i,j), trichoplein was not concentrated at NF or desmin-positive IFs. In addition, we found that the blood vessels are trichoplein-positive (d-f). (C) Vertical view (k) and horizontal view (l) of hair follicle stained with trichoplein antibody. Trichoplein localized at the outer root sheath (arrowheads in k,l), where K6/16 is found. (D) Double staining of the brain with trichoplein and PECAM-1 antibodies (m-o). Trichoplein localized to the blood vessels where PECAM-1 was also positive. Bar, 10 μm.

In HeLa cells, trichoplein is associated with K8/18 filament bundles, especially in the cell periphery (Fig. 5). Trichoplein does not bind along the entire keratin filaments but is concentrated in a dot-like staining pattern. Therefore, it appears that trichoplein could act as a 'capping' or 'branching' protein for keratin filaments in the cell periphery. On the other hand, in polarized Caco-2 cells and the enterocytes of the small intestine, trichoplein colocalizes with K8/18 filaments beneath the apical membrane, and is also concentrated at desmosomes (Figs 6, 8). It is well known that a network of IFs, the major components of the terminal web, bridges desmosomes under the apical membrane in brush border cells (Franke et al., 1979; Hirokawa et al., 1982), although the molecular mechanisms underlying the formation of this structure remain unclear. Salas and colleagues described the existence of a thin layer (a terminal web-like structure) of keratin IFs located beneath the apical membrane of a variety of simple epithelial cells (Rodriguez et al., 1994; Salas et al., 1997), and suggested that this apical network of keratin IFs participates in the apical plasma membrane polarity, including the organization of apical microfilaments and microtubules (Salas et al., 1997; Salas, 1999). Consistent with this hypothesis, anomalous apical plasma membrane phenotypes were observed in K8-deficient mice (Ameen et al., 2001). Taken together with these observations, it is tempting to speculate that trichoplein may regulate K8/18 filament organization mainly at the apical or peripheral regions of epithelial cells, and might affect cell polarity. Although HeLa cells contain desmosomes, endogenous trichoplein and GFP-trichoplein are not concentrated at desmosomes of HeLa cells even in confluent conditions. This suggests that the association of trichoplein with desmosomes might require a cell-type specific (e.g. intestinal cell-specific or simple epithelia-specific) desmosomal protein. In addition, because trichoplein is expressed not only in epithelial tissues but also in non-epithelial tissues where keratins are absent (Fig. 7), trichoplein may have other functions independent of keratin IFs.

Proteins that show homology to trichohyalin and plectin,

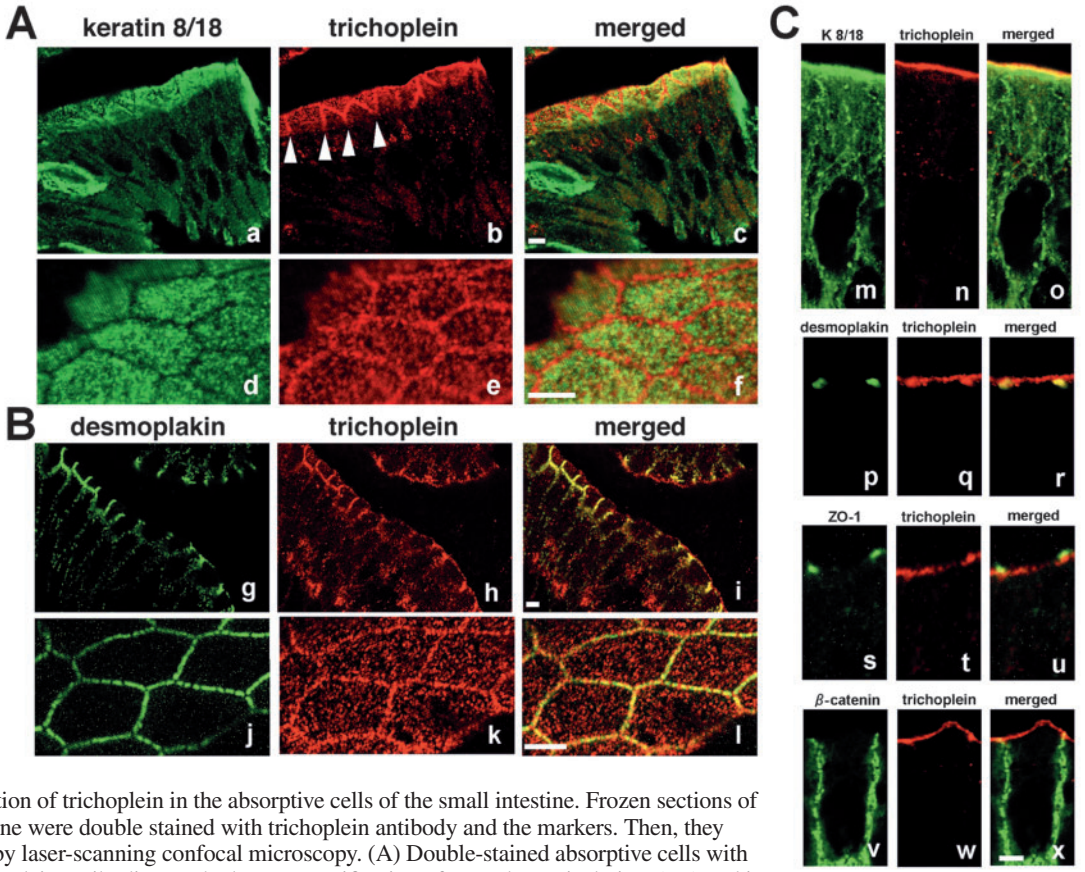


Fig. 8. Distribution of trichoplein in the absorptive cells of the small intestine. Frozen sections of the small intestine were double stained with trichoplein antibody and the markers. Then, they were analyzed by laser-scanning confocal microscopy. (A) Double-stained absorptive cells with K8/18 and trichoplein antibodies. In the lower magnification of a nearly vertical view (a-c) and in the higher magnification of a horizontal view (d-f), trichoplein was associated with K8/18 in the apical region (terminal web). Panel e and arrowheads in b show trichoplein concentrating at the cell-cell border. (B) Double-stained absorptive cells with desmoplakin and trichoplein antibodies. In the lower magnification image of a nearly vertical view (g-i) and the higher magnification of a horizontal view (j-l), trichoplein is closely associated with desmoplakin at the cell-cell border. (C) Higher magnification of the vertical sectional views of the absorptive cells double stained with trichoplein antibody and K8/18 antibody or antibodies for the markers of junctional complex (ZO-1, β-catenin or desmoplakin 1&2). Trichoplein is located close to K8/18-positive signals of higher density in the apical region (m-o). At the cell-cell border, trichoplein closely associates with desmoplakin (p-r), and does not always merge with ZO-1 (s-u) or β-catenin (v-x). These observations are similar to those of Caco-2 cells in Fig. 6. Bar, 10 μm.

AJM-1 (Köppen et al., 2001) and Fbf-1 (Schmidt et al., 2000) have been reported. AJM-1 is a coiled-coil protein localizing to an apical junctional domain of *Caenorhabditis elegans* epithelia basal to the HMR-HMP (cadherin-catenin) complex. In the absence of AJM-1, the integrity of this domain is compromised (Köppen et al., 2001). Fbf-1 was identified to be a novel protein that bound to the cytosolic domain of the murine CD95/APO-1/FAS, a cell surface receptor mediating programmed cell death in response to various stimuli (Schmidt et al., 2000). At present, it is completely unclear whether trichoplein, AJM-1 and Fbf-1 have a common physiological function as family proteins that show homology to trichohyalin and plectin.

To determine whether trichoplein plays a role in K8/18 filament organization, desmosomes or cell polarity, we knocked down trichoplein in cultured cells using an siRNA technique, but thus far we have observed no apparent phenotypes (data not shown). Therefore we are now preparing trichoplein knockout mice, which should shed light on this problem.

In conclusion, we identified trichoplein as a keratin-biding protein, which may be involved in the organization of the apical network of keratin filaments and desmosomes in simple epithelial cells. These observations will pave the way toward further research on keratin filament organization as well as identifying the pathological mechanisms of keratin-related diseases.

We thank H. Eto (Massachusetts General Hospital, Boston, MA), E. Fuchs (The University of Chicago, Chicago, IL) and P. James (The University of Wisconsin, Madison, WI) for kindly providing plasmids; R. Liem and C. Leung (Columbia University College of Physicians and Surgeons, New York, NY) for providing yeast vectors; Sh. Tsukita (Kyoto University, Kyoto, Japan) for providing mouse anti-ZO-1 monoclonal antibody and T. Itoh (Nagoya University, Nagoya, Japan) for providing purified tubulin and actin. We are grateful to M. Ohara (Fukuoka) for language assistance and critical comments on the manuscript. This work supported in part by Grants-in-Aid for Scientific Research and Cancer Research from the Ministry of Education, Science, Technology, Sports, and Culture of Japan, by a grant-in-aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan, by The Naito Foundation, and by the Uehara Memorial Foundation.

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