Characterization of Pinin, A Novel Protein Associated with the Desmosome–Intermediate Filament Complex

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Abstract. We have identified a protein named pinin that is associated with the mature desmosomes of the epithelia (Ouyang, P., and S.P. Sugrue. 1992. *J. Cell Biol.* 118:1477–1488). We suggest that the function of pinin is to pin intermediate filaments to the desmosome. Therefore, pinin may play a significant role in reinforcing the intermediate filament–desmosome complex. cDNA clones coding for pinin were identified, using degenerative oligonucleotide probes that were based on the internal amino acid sequence of pinin for the screening of a cDNA library. Immunoblotting of expressed recombinant proteins with the monoclonal 08L antibody localized the 08L epitope to the carboxyl end of the protein. Polyclonal antibodies directed against fusion proteins immunoidentified the 140-kD protein in tissue extracts. Immunofluorescence analysis, using the antifusion protein antibody, demonstrated pinin at lateral epithelial boundaries, which is consistent with desmosomal localization. The conceptual translation product of the cDNA clones contained three unique domains: (*a*) a serine-rich domain; (*b*) a glutamine-proline, glutamine-leucine repeat domain; and (*c*) an acidic domain rich in glutamic acid. Although the 3′ end of the open reading frame of the clone for pinin showed near identity to a partial cDNA isolated for a pig neutrophil phosphoprotein (Bellavite, P., F. Bazzoni, et al. 1990. *Biochem. Biophys. Res. Commun.* 170:915–922), the remaining sequence demonstrated little homology to known protein sequences. Northern blots of mRNA from chicken corneal epithelium, MDCK cells, and various human tissues indicated that pinin messages exhibit tissue-specific variation in size, ranging from 3.2 to 4.1 kb. Genomic Southern blots revealed the existence of one gene for pinin, suggesting alternative splicing of the mRNA. Expression of the full-length cDNA clones in human 293 cells and monkey COS-7 cells demonstrated that a 140-kD immunoreactive species on Western blots corresponded to pinin. Pinin cDNA transfected into the transformed 293 cells resulted in enhanced cell–cell adhesion. Immunofluorescence staining revealed that the expressed pinin protein was assembled to the lateral boundaries of the cells in contact, which is consistent with the staining pattern of pinin in epithelial cells.

D ESMOSOMES (*Macula adherens*) are intimately involved in the structural and functional integration of adjacent epithelial cells. They serve as reinforcement sites of cell–cell adhesion, as well as points for lateral anchorage of the intermediate scaffold of the epithelial cell (Staehelin, 1974; Arnn and Staehelin, 1981). Ultrastructurally, they appear as symmetrically arranged disc-shaped structures of a varying diameter (0.1–2 μm). The space between the interacting membranes is 20–30 nm, which often exhibits a central electron-dense core, presumably consisting of the overlapping domains of the transmembrane glycoproteins of the desmosome. On each cytoplasmic side of the interacting membranes, there are trilaminar plaques that appear to anchor the looping bundles of intermediate filaments (IF[1]; for reviews see Buxton and Magee, 1992; Buxton et al., 1993; Garrod, 1993; Legan et al., 1992). Biochemical and molecular analyses have led to the identification of several constitutive proteins, including desmoplakin, plakoglobin, and the transmembrane cadherin-like glycoproteins desmoglein and desmocollin. (Mueller and Franke, 1983; Cowin et al., 1985 1986; Green et al., 1990; Holton et al., 1990; Collins et al., 1991; Wheeler et al., 1991; Wiche et al., 1991; Green et al., 1992). Significant differences in the composition of desmosomes of various tissues have also been reported. Isoforms of desmoglein (Dsg1-3) and desmocollin (Dscl-3) have been

1. *Abbreviations used in this paper:* GST, glutathione *S*-transferase; IF, intermediate filament; MDBK, Madin-Darby bovine kidney (cells).

found in several desmosome-containing tissues and between layers of the same tissue (Parrish et al., 1986; Angst et al., 1990; Koch et al., 1991; Legan et al., 1992; Arnemann et al., 1993; Buxton et al., 1993; Theis et al., 1993). Other desmosomal plaque–associated molecules have been reported in limited subsets of epithelial tissues. These include a shorter spliced form of desmoplakin (desmoplakin II; Mueller and Franke, 1983; Cowin et al., 1985; Angst et al., 1990; Green et al., 1990); plakophilin (formerly band-6-protein), a "new" member of the plakoglobin/armadillo gene family (Hatzfeld et al., 1994; Heid et al., 1994); desmocalmin, a Ca^{++}-binding protein (Tsukita and Tsukita, 1985); plectin, the large IF associated protein (Wiche et al., 1991; Wiche et al., 1993); and IFAP 300 (Skalli et al., 1994).

Although many of the molecular constituents of the desmosome have now been characterized, key questions remain concerning the molecular organization of the desmosome, the mechanism of desmosomal assembly and disassembly, and the modulation of the desmosome during essential activities of the epithelial cell.

We have identified a phosphoprotein with an $M_r \sim 140,000$, as judged by SDS-PAGE and Western blotting, which was found to be associated with all mature desmosomes (Ouyang and Sugrue, 1992). This molecule, which was identified by mAb 08L, is now referred to as pinin. The 08L antibody stained the intracellular side of lateral epithelial cell margins near the cytoplasmic face of the desmosomal complex in the vicinity of intermediate filament convergence onto the desmosome. The 08L antigen did not localize to the desmosomal plaque proper; rather, it was localized to the periphery of the plaque. Examination of the assembly of pinin to desmosomal complexes in cells grown at low confluence or in low calcium conditions revealed the pinin to be recruited to preformed, morphologically identifiable desmosomes. The presence of 08L immunoreactivity at the desmosome correlated with the establishment of a highly organized desmosome-IF complex. These observations led us to conclude that the 08L protein was not integral to the desmosome proper, but rather may be involved in the organization and/or stabilization of the more mature or definitive desmosome-IF complex.

Here, we present data regarding the purification, molecular cloning, and expression of pinin. Sequence analysis of cDNA clones suggests that pinin is a new protein with little or no overall homology to other desmosomal or IF-associated proteins. Northern blot analyses and the identification of pinin immunoreactivity within nerve cells tempt us to speculate that pinin may represent one family of molecules involved in IF membrane assemblies. Results from transfections of pinin cDNA suggest a key role for pinin in the stabilization of epithelial cell–cell adhesion.

Materials and Methods

Reagents

DME and FCS were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Hanks' medium and other supplements for cell culture, unless otherwise described, were purchased from Irvine Scientific (Santa Ana, CA). PMSF, leupeptin, chemostatin, and pepstatin were purchased from Sigma Chem. Co. (St. Louis, MO). The MDCK cell line was kindly provided by Dr. Karl Matlin (Harvard Medical School, Boston, MA). All molecular bi-

ology reagents, including restriction enzymes, were purchased from Boehringer Mannheim Corp. (Indianapolis, IN), unless otherwise stated.

Cell Culture

MDCK cell line of passages 10-60, human 293 transformed embryonic kidney epithelial cells, and COS-7 African Green monkey kidney cells that constitutively express SV-40 large T antigen were maintained in DME and supplemented with 10% FCS, 2 mM glutamine, and 200 U/ml each of streptomycin and penicillin G. Cells were passed with 0.1% trypsin and 0.04% EDTA in Hanks' medium.

Purification of Pinin

MDCK cells were sequentially extracted with CSK buffer (10 mM Pipes, 300 mM sucrose, 150 mM NaCl, 3 mM $CaCl_2$, 1 mM EDTA, 0.1 mM DTT, 0.5% Triton X-100, pH 6.8, 1 mM PMSF, 1 μg/ml each of pepstatin, leupeptin and chemostatin), and 1.5 M KCl in 10 mM Tris, pH 7.4. The KCl-soluble fractions were dialyzed extensively against 10 mM Tris, pH 7.4, followed by centrifugation. The precipitate contained >90% of pinin based on immunoblotting. Then 10 mM Tris containing 7 M urea was added to the precipitate, which dissolved pinin. The urea-soluble fraction was then filtered through a 0.22-μm filter and subjected to gel filtration on a 1.5× 120–cm Sephacryl-400 column (Pharmacia LKB Biotechnology, Piscataway, NJ) with a flow rate of 5 ml/h. Fractions containing pinin were pooled and applied to a DEAE filter disk (FMC Corp. BioProducts, Rockland, ME). Protein bound to DEAE support was eluted with a linear salt-gradient from 0.1 to 0.5 M NaCl, following washing in 0.1 M Tris with 0.05 M NaCl. The 08L-positive fractions were subsequently identified by immunoblotting and were pooled. Pooled fractions were concentrated with filters (Centricon; Amicon, Beverly, MA). Samples were resolved by 6% SDS-PAGE and transferred to nitrocellulose filter paper. The band corresponding to pinin was excised and prepared for trypsin digestion and microsequencing (Bill Lane, Microchemistry Laboratory, Harvard University).

Screening of cDNA Libraries

An oriented MDCK cDNA library constructed in UNI-ZAP XR vector (Stratagene, La Jolla, CA) was kindly provided by Dr. Marino Zerial European Molecular Biology Laboratory (EMBL) Heidelberg, Germany. This library was screened with an oligonucleotide probe (po36) based on the amino acid sequence derived from one of the tryptic fragments (36,V-E-L-A-Q-L-Q-E-E-W-N-E-H-N-A-K). The sequence of the 256-fold degenerate oligonucleotide probe po36 was as follows: 5'-GTTGA(A/G)(C/T)TTGC(C/A)GCTTCAGGA(A/G)GA(A/G)TGGAA (T/C)GA(A/G)CA(T/C)AA(T/C)GCIAA-3'. A total of 300,000 phage plaques were screened. Duplicate filters were prehybridized at 60°C overnight in 6× standard saline citrate (SSC), 1× SSPE, 2× Denhardt's solution, and 0.25% SDS containing 100 μg/ml boiled salmon sperm DNA. Hybridization was performed under the same conditions as prehybridization with the addition of polynucleotide kinase ^{32}P-labeled po36. Filters were then washed for 1 h at 60°C with 2× SSC and 0.05% SDS, and were then exposed to X-OMAT film (Eastman Kodak Co., Rochester, NY). The cDNA library was rescreened with the random-primed ^{32}P-labeled 1.6-kb EcoR1 fragment of po36-5. Filters to be probed with the DNA fragment were prehybridized in 50% formamide, 5× SSPE, and 5× Denhardt's solution with 100 μg/ml salmon sperm DNA. Hybridization was carried out for 18 h in the same solution at 60°C. Filters were washed four times in 0.2 × SSC and 0.05% SDS at 60°C for 15 min.

A human placenta cDNA library, which was oligo(dT) and random primed (HL3007b; CLONTECH, Palo Alto, CA), was used to isolate clones sshp6A and sshp6B via screening with MDCK clone ss13. In addition, a bovine kidney cell line (MDBK) library (BL3001b, Clontech, Palo Alto, CA) was used to identify clones bk5 and bk16.

5' RACE of MDCK cDNA

MDCK cell total RNA was prepared according to the single-step method (Chomczynski and Sacchi, 1987). First-strand cDNA was constructed in the presence of Superscript reverse transcriptase (Stratagene) by priming total RNA with a specific primer gsp13 located 230 bp downstream from the 5' end of ss13. Single-strand ligation of cDNAs with an oligonucleotide anchor was performed using T4 ligase at 22°C overnight. The ligation products were then used as templates for PCR. PCR was carried out for 35 cycles consisting of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min.

Reactions were primed with the nested ss13-specific primer, gsp23, which is located 110 bp upstream of gsp13, and a primer complementary to the anchor sequence. The PCR product was confirmed by Southern blot and sequencing.

5' end–anchored human placenta DNA purchased from Clontech (5' RACE Ready cDNA) was used to generate the 5' end of human pinin by the same procedure described above.

DNA Sequencing

cDNA inserts were rescued from the UNI-ZAP XR vector according to the manufacturer's protocol (Stratagene). Inserts from gt11-derived clones were excised from λ DNA preps and ligated into pBluescript SK(+). Phagemid DNA, prepared by Magic miniprep (Promega, Madison, WI), was used directly for double-stranded DNA sequencing with Sequenase II (United States Biochem Corp., Cleveland, OH) using universal primers and then a series of selected primers 17–18 nucleotides in length. The selection of primers was based on GC content and location ~50 nucleotides proximal to termination of previous sequence. (The MDBK cDNAs were sequenced at the DNA sequencing core of University of Florida Interdisciplinary Center for Biotechnology Research).

Expression and Purification of Recombinant Pinin

cDNA synthesis was carried out by priming the ss13 template with specific oligonucleotides linked to sequence of the restriction enzyme sites. BamH1 was used on sense primers and EcoR1 was used on antisense primers (lowercase below). Sequences were selected to generate polypeptides between 10 and 14 kD. Peptide 1 spanning amino acid residues 11–111 was generated with sense primer (ctctcggatcccAATATTCGCAAGCT-CACC) and antisense primer (gggaattcccgACGTGTGCGCTCTTTG-GAG); peptide 2, amino acids 187–303, was generated with sense primer (ctctcggatcccAAACAGACAGAACTGCGG) and antisense primer (gg-gaattcccgTTCCTCTCGCTGAGCCAC); peptide 3, amino acids 452–580, was generated with sense primer (ctctcggatcccAGAGAATCTGAGCCC-CAG) and antisense primer (gggaattcccgTTTGCTATCTGAATGGAC); and peptide 4, amino acids 566–702, was generated with sense primer ctctcggatcccCTGAGGTGACAGAGAGCC and antisense gggaattcccgGAT-GTATCCCTTCGTTCCG. An additional, larger peptide 12, amino acids 452–702 was generated by priming with the sense primer of peptide 3 and antisense primer of peptide 4. The PCR was performed for 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using Vent^R DNA polymerase (New England Biolabs Inc., Beverly, MA). PCR products were resolved on 1% agarose, 1% NuSieve gels, excised and cleaned, then digested with restriction endonucleases BamHI and EcoRI and ligated into the pGEX-1 vector, which contains the carboxyl terminus of glutathione S-transferase (GST), 27.5 kD, under control of the tac promoter. The bacteria that were transformed successfully with recombinant vector and verified by the restriction digestion of plasmid minipreps were induced with IPTG and 0.1 mM in Luria broth with ampicillin for 3 h at 37°C. Bacteria pelleted from 1.5 ml of the culture were then lysed in sample buffer and boiled for 3 min, and were applied to 10% SDS-PAGE gels. Purifications of expressed proteins were accomplished by absorption of fusion proteins from bacterial cell lysates to glutathione-Sepharose, followed by elution with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0.

Antibody Production

Fusion protein containing peptide 3 was used as the immunogen. Polyclonal antibodies were produced in rabbits by BABCO. (Berkeley Antibody Co., Berkeley, CA). The initial inoculation, containing 300 μg of fusion protein and four boost injections of 120 μg, was given at 3-wk intervals. 2 wk later, final boost rabbit serum was harvested. Western blot and immunofluorescence analyses were carried out with the reactive and preimmune sera.

Northern Blots

RNA was prepared by disruption of embryonic day 18 chick corneal epithelia and MDCK cells in 6 M guanidinium isothiocyanate, followed by centrifugation through cesium chloride. Poly(A) RNA was isolated by chromatograph over oligo-(dT) cellulose. RNA, 5 μg poly-A-RNA or 8 μg of total RNA (MDCK), were separated by electrophoresis through 2.2 M formaldehyde and 1.0% agarose and blotted to nylon paper (Hybond; Amersham, Arlington Heights, IL). These filters and human multiple tissue Northern blot filters (CLONTECH) were prehybridized in 50% for-

mamide, 5× SSC, 5× Denhardt's solution, 0.2% SDS, and 200 μg/ml salmon sperm DNA for 10 h at 42°C. Next, they were hybridized in the same solution with ^{32}P-labeled 1.6-kb EcoRI fragment or 280-bp EcoRI-AccI fragment of ss13 at 42°C for 16 h. Filters were then washed twice in 2× SSC with 0.1% SDS for 30 min at room temperature and 1× SSC with 0.1% SDS at 55°C for 2 h, and were exposed to Kodak XAR-5 film at −80°C with an intensifying screen.

Genomic Southern Blots

Genomic DNA was isolated from human peripheral blood. The genomic DNA was then digested with restriction enzyme EcoRI, HindIII, or PstI, and was transferred to nitrocellulose. After prehybridization incubation, the blots were probed with the 1.5-kb EcoRI fragment of the human cDNA sshp(6A).

Construction of Mammalian Expression Vectors and Transfections

Full-length cDNA with 5' untranslated region stretch of 11 bp (CA-GAGAGAAGATG...) was ligated into pCDNA3 (Invitrogen, San Diego, CA). The fidelity of products was confirmed by DNA sequencing using internal and flanking primers. DNA was transfected into monkey kidney-derived COS-7 cells and 293 cells (an embryonic kidney cell line) using the calcium phosphate method (Graham and Eb, 1973). Positive transfectants were selected with G418 (0.6 mg/ml effective concentration; GIBCO BRL, Gaithersburg, MD).

Preparation and Immunoblotting of Whole-cell Extracts

MDCK cells, stable transfectants containing pinin DNA, as well as control vector DNA transfectants and nontransfectants, were extracted as described by Ouyang and Sugrue, 1992. Samples containing 30 μg of protein were loaded and run on 8% SDS-polyacrylamide gels. The gels were transferred to nitrocellulose and immunoblotting was carried out as described previously (Ouyang and Sugrue, 1992). The primary antibodies were used at dilutions of 1:1,000 of polyclonal serum 3A and 1:10 for ammonium sulfate–purified 08L mAb. Primary antibodies were detected by a 1:1,000 dilution of peroxidase-coupled secondary antibody (Boehringer Mannheim, Indianapolis, IN). The peroxidase was then visualized by 0.5 mg/ml diaminobenzidine or ECL reagent (Amersham).
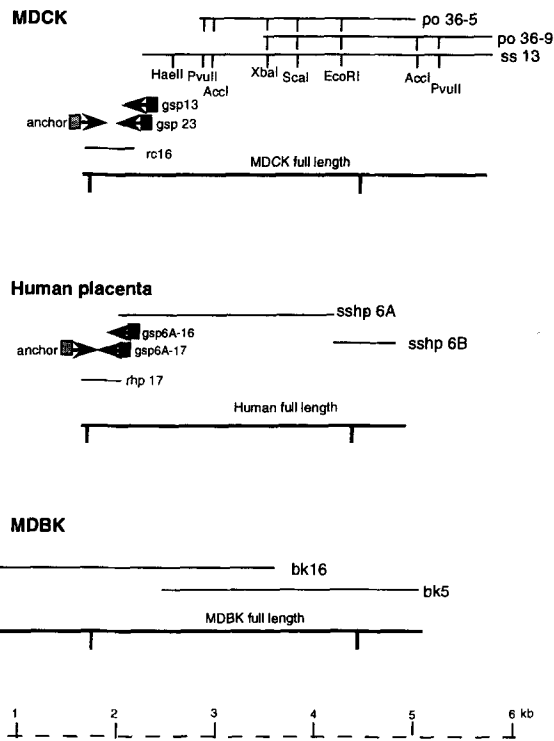
Immunofluorescence

For immunohistochemistry, cells were grown on glass coverslips. Cells were washed in PBS and fixed in −20°C acetone for 2 min. After washes in PBS, cells were incubated in primary antibodies: mAb (08L) hybridoma supernatant was used at 1:20 dilution and polyclonal m3A was diluted 1:200. Desmoplakin multiepitope antibody cocktail was used to visualize desmoplakin (DP 2.15, DP 2.17, DP 2.20 IgG1; American Research Products, Inc., Belmont, MA). Rat mAb to ZO1 (R40.76) was generously provided by Dr. Dan Goodenough (Department of Cell Biology, Harvard Medical School). Primary incubations were carried out for 1 h at room temperature. Secondary antibodies were used at a dilution of 1:200: goat anti–mouse and goat anti–rabbit conjugated to either FITC (Boehringer Mannheim) or to Texas red (Cappel Organon Teknika Corp., West Chester, PA). Controls included the incubation of fixed cells in preimmune rabbit serum for polyclonal antibody and conjugated secondary antibodies only.

Results

Identification of cDNA for Pinin

We previously showed that unlike the majority of characterized desmosomal proteins, pinin is extractable in high salt–containing buffers (Ouyang and Sugrue, 1992). Western blots of two-dimensional gels revealed the existence of multiple isoforms of pinin, with isoelectric point ranging from 5.9 to 6.4. Taking advantage of the solubility property and the observed pI of pinin, we purified pinin through the use of differential extractions, standard chro-

a

MDCK

po 36-5
po 36-9
ss 13

HaeII PvuII
AccI
XbaI ScaI EcoRI AccI PvuII

◀ gsp13
anchor ▶ ◀ gsp 23
rc16
MDCK full length

Human placenta

sshp 6A
gsp6A-16
anchor ▶ ◀ gsp6A-17
sshp 6B
rhp 17
Human full length

MDBK

bk16
bk5
MDBK full length

1   2   3   4   5   6 kb

b

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1/1
CTC TGA AGG TTC CAG AAT CGA TAG TAA AGT CTC GAA GCC TGC TGC AGA GAG AAG ATG GCG
                                                                          M   A
61/3
GTC GCG GTG AGA ACT TTG CAG GAG CAG CTA GAA AAG GCC AAA GAG AGC CTA AAG AAC GTG
V   A   V   R   T   L   Q   E   Q   L   E   R   A   K   E   S   L   K   N   V
121/23
GAT GAG AAT ATT CGC AAG CTC ACC GGA CGG GAT CCG AAT GAC GTA AGG CCC ATC CAA GCC
D   E   N   I   R   K   L   T   G   R   D   P   N   D   V   R   P   I   Q   A
181/43
AGA TTG CTG GCC CTT TCT GGT CCT GGT GGA GGT AGA GGA CGT GGT AGT TTA TTG CTG AGG
R   L   L   A   L   S   G   P   G   G   G   R   G   R   G   S   L   L   L   R
241/63
CGT GGA TTC TCA GAT AGT GGA GGA GGA CCC CCA GCC AAA CAG AGA GAC CTT GAA GGG GCA
R   G   F   S   D   S   G   G   G   P   P   A   K   Q   R   D   L   E   G   A
301/83
GTC AGT AGG CTG GGC GGG GAG CGT CGG ACA AGG AGA GAA TCA CGC CAA GAA AGC GAC CCA
V   S   R   L   G   G   E   R   R   T   R   R   E   S   R   Q   E   S   D   P
361/103
GAG GAT GAT GAT GTT AAA AAG CCA GCT TTG CAG TCT TCA GTT GTA GCT ACC TCC AAA GAG
E   D   D   D   V   K   K   P   A   L   Q   S   S   V   V   A   T   S   K   E
421/123
CGC ACA CGT AGA GAC CTT ATC CAG GAT CAA AAT ATG GAT GAA AAG GGA AAG CAA AGG AAC
R   T   R   R   D   L   I   Q   D   Q   N   M   D   E   K   G   K   Q   R   N
481/143
CGA CGA ATA TTT GGC TTA TTG ATG GGC ACT CTT CAG AAA TTT AAA GAA GAA TCC ACT GTT
R   R   I   F   G   L   L   M   G   T   L   Q   K   F   K   Q   E   S   T   V
541/163
GCT ACT GAA AGG CAA AAG AGG CGC CAG GAA ATT GAA CAA AAA CTT GAA GTG CAG GCG GAA
A   T   E   R   Q   K   R   R   Q   E   I   E   Q   K   L   E   V   Q   A   E
601/183
GAA GAA AGA AAG CAG GTT GAA AAT GAG AGG AGA GAA CTG TTT GAA GAG AGG CGT GCT AAA
E   E   R   K   Q   V   E   N   E   R   R   E   L   F   E   E   R   R   A   K
661/203
CAG ACA GAA CTG CGG CTT TTA GAA CAG AAG GTT GAG CTT GCG CAG CTG CAA GAA GAA TGG
Q   T   E   L   R   L   L   E   Q   K   V   R   L   A   Q   L   Q   R   E   W
721/223
AAT GAA CAT AAT GCC AAG ATA ATT AAA TAC ATA AGA ACT AAG ACA AAG CCC CAT TTG TTC
N   E   H   N   A   K   I   I   K   Y   I   R   T   K   T   K   P   H   L   F
781/243
TAT ATT CTC GGA AGA ATG TGT CCA GCT ACC CAA AAA TTA ATA GAA GAG TCA CAG AGA AAA
Y   I   L   G   R   M   C   P   A   T   Q   K   L   I   E   E   S   Q   R   K
841/263
ATG AAT GCT TTA TTT GAA GGT AGA CGC ATC GAA TTT GCA GAA CAA ATA AAT AAA ATG GAG
M   N   A   L   F   E   G   R   R   I   E   F   A   E   Q   I   N   K   M   E
901/283
GCT AGG CCT AGA AGA CAA TCA ATG AAG GAA AAA GAG CAT CAG GTG GTG CGT AAT GAA GAA
A   R   P   R   R   Q   S   M   K   E   K   E   H   Q   V   V   R   N   E   E
961/303
CAG AAG GCG GAA CAA GAA GAG GGT AAG GTG GCT CAG CGA GAG GAA GAG TTG GAG GAG ACA
Q   K   A   E   Q   E   E   G   K   V   A   Q   R   E   E   E   L   E   E   T
1021/323
GGT AAT CAG CAC AAT GAT GTA GAA ATA GAG GAA GCA GGA GAG GAA GAG GAA AAG GAA ATA
G   N   Q   H   N   D   V   E   I   E   E   A   G   E   E   E   E   K   E   I
1081/343
GGT ATA GTT CAT AGT GAT GCA GAG AAA GAG GAG GAG GAG GAG GAA CAA AAA CAG GAA ATG
G   I   V   H   S   D   A   E   K   E   Q   E   E   E   E   Q   K   Q   E   M
1141/363
GAG GTC AAG ATC GAG GAG AAA ACT GAG GTA AGG GAA AGT GAG AAG CAG CAG GAT AGT CAA
E   V   K   I   E   E   E   T   E   V   R   E   S   E   K   Q   Q   D   S   Q
1201/383
CCG GAA GAA GTT ATG GAT GTG CTA GAG ATG CTG TTG CAC GTA GCT GTC AAA AAT GTA ATT
P   E   E   V   M   D   V   L   E   M   L   L   H   V   A   V   K   N   V   I
1261/403
GCT GAG CAG GAG GTA ATG GAA ACC AAT CAA GTT GAA AGT GTA GAA CCT TCA GAA AAT GAA
A   E   Q   E   V   M   E   T   N   Q   V   E   S   V   E   P   S   E   N   E
1321/423
ACT AGC AAA GAA CTG GAG CCA GAA ATG GAA TTT GAA GTT GAG CCA GAT AAA GAA TGT AAA
T   S   K   E   L   E   P   E   M   E   F   E   V   E   P   D   K   E   C   K
1381/443
TCT CTT TCT CCT GTG AGA GAG AAT GCC AGT GCT CTA GAA ATG GAA AAT GAG CCT GAG GAA
S   L   S   P   V   R   E   N   A   S   A   L   E   M   E   N   E   P   E   E
1441/463
AAA GAA GAG AGA GAA TCT GAG CCC CAG CCT GAG CCT GTG CGT CAC CTC CAG CCC TTG CCT
K   E   E   R   E   S   E   P   Q   P   E   P   V   R   H   L   Q   P   L   P
1501/483
CAG CCT GAG CCT GAG CCT GAG CTA CAG CCT GAG CCA CAG CCT CAG CTA CAA CCT GAG CCA
Q   P   E   P   E   P   E   L   Q   P   E   P   E   P   Q   L   Q   P   E   P
1561/503
CAG TTA CAG CCC CAG CTC CAG CTC CAG CTC CAG CCC CAG CCC CAA TCC CAG TCC CAG CCA
Q   L   Q   P   Q   L   Q   L   Q   L   Q   P   Q   P   Q   S   Q   S   Q   P
1621/523
CAA CCT CAG CTT CAG CTT CCG CTT CCT CTT CCG CTT CAG CCC CAG CCC CAG GTG CAG GCC
Q   P   Q   L   Q   L   P   L   P   L   P   L   Q   P   Q   P   Q   V   Q   A
1681/543
CAG TCC CAG CCC CAA GCA GTA CTC CAA CCC CAG CCT GTC TCT CAA CCT GAG ACT TTG CCA
Q   S   Q   P   Q   A   V   L   Q   P   Q   P   V   S   Q   P   E   T   L   P
1741/563
TTA GCT GTT CTA CAG GCA CCA GTT CAG GTT ATT CAA GAG CAA GGG CAT TTA CTA CCT GAG
L   A   V   L   Q   A   P   V   Q   V   I   Q   E   Q   G   H   L   L   P   E
1801/583
AGG AAG GAA TTT CCT GTG GAA TCT GTA AAA CTC ACT GAG GTG ACA GTA GAG CCA GTC TTG
R   K   E   F   P   V   E   S   V   K   L   T   E   V   T   V   E   P   V   L
1861/603
ATA GTA CAT TCA GAT AGC AAA ACC AAA ACC AAA ACC AGG AGC AGA AGT AGA GGT CGA GCT
I   V   H   S   D   S   K   T   K   T   K   T   R   S   R   S   R   G   R   A
1921/623
AGA AAT AAA ACC AGC AAG AGT AGA AGT CGC AGC AGT AGC AGT AGT AGT TCT AGT AGC AGC
R   N   K   T   S   K   S   R   S   R   S   S   S   S   S   S   S   S   S   S
1981/643
TCA ACC AGT AGC AGC AGT GGA GGC AGT TCC AGC AGT GGC AGC AGT AGT AGT CGA AGT AGT
S   T   S   S   S   G   G   S   S   S   S   S   S   S   S   S   R   S   S
2041/663
TCC AGT AGC AGC TCC AGC ACA AGT GGC AGC AGC AGG AGA GAT AGT AGC AGC AGC ACT ACT
S   S   S   S   S   S   T   S   G   S   S   R   R   D   S   S   S   S   T   T
2101/683
AGT AGC AGC GAG AGT AGA AGT CGG AGT AGG AGC CGG GGA CAT AAC AGA GAT AGA AAG CAC
S   S   S   E   S   R   S   R   S   R   G   R   G   H   N   R   D   R   K   H
2161/703
AGA AGG AGC GTG GAT CGG AAA CGA AGG GAT ACA TCA GGA TTA GAA AGA AGT CAC AAA TCT
R   R   S   V   D   R   K   R   R   D   T   S   G   L   E   R   S   H   K   S
2221/723
TCA AAA CGT GGT AGT AGT AGA GAT ACA AAA GGA TCA AAG GAT AAG AAT TCC CGG TCC GAC
S   K   G   S   S   R   D   T   K   G   S   K   D   K   N   S   R   S   D
2281/743
AGA AAG AGG TCT ATA TCA GAG AGT AGT CGA TCA GGC AAA AGA TCT TCA AGA AGT AGA AGA
R   K   R   S   I   S   E   S   S   R   S   G   K   R   S   S   R   S   R   R
2341/763
GAC CGA AAA TCA GAC ACG AAA GAC AAA AGG CGT TAA TGG AAG AAG CCA GGC TTT CTT AGC
D   R   K   S   D   R   K   D   K   R   R   *
CTA TTC TTT GCC GCA GAA GAT TTC TTG ATG AGT AAA AGG ATT ACC TTT CCT TGT AAG GAG
GAT GCT GCC TTA AGA ATT GCA TGT TGT AAA AAA TCT TTT TTG GAA AAT ACA GAC TGT TTG
TTT ACC AGA CAT TCT TGT ACT TTT TGC ATA ATT TTG TAA GAG TTA TTT ATC AAA ATT ATG
TGA GGT TCC AAA ATA TGT AAA AAT TAT AAT AAT AAA AAA AGA TTA ACA TCC CTT GTC ATC
TTT TTT AAA TAT CCT ATA CAC TTC AGT AAG AAT CTG TAT ATT TTA ATA GGT AAA TCT TTA
CGC TCC TGT TCC CTT CAA ATT CTG TAT CAT ACA TTG CTT TTC GTC AGA AAT AAA TCG TGC
ATT TCT TTC ATT AGT TTT CGG AAT CGT CCT CCG TTG ACA CTT GTA TAA TAA ATT ACC CTC
TTG ATA TAT CGT TTT CAG CTT TTA TCA CTA GAT ACT GAA CGT GAT TAG AAT GTC TTT GAA
GGG TTG ATT ACT ACT AAT CAA CTA TTG TCG TCT TTG TAT GGA AGA AAA TAA TAA AAT AGT
TGG TCG ACT ATT CTT CTT CTA CTA TAT GAT GTT TGT GAG AAT AGT TGC TCC TCC TGC TGT
GTA GCA ACC ATC TTG TTG ATT AAT AAT AAT AAA ATA TTA TCA CCC TCC TTC TGA GTA ATT
ACT ACT ACT ATT TCA GAC GTC TTT ATT ACT ACA TCA TGA AGT AAT CAT CGT AGA ACA GCT
GGT AGT ATA TGA AGA ATA GTT GCT CTT TTT TAT ATG AGA AAC GCG GGG AAA TGC TTA GAT
TAA AAC AAG GGA GAT TAG AAA AAA ATA GTA AAT GAT TCT GAG AAC AAA CTA TGT TCC CCA
AAA GCG TGC AAA GAT CTG ACT ATG ACA AGC TCC AAA TAA TTG CCT AAG GCA AAT AAA AGT
GAG GAA ACC TTC AAA TGA CAT TCT AAT CTA CTG TTC AGT ATC TAG TGA TAA ACG TCG GAA
CAT AGT TTG TTC TCA GAA TCA TTT ACT ATT TTT TCT AAT CTC CCT TGT TTT AAT CTA AGC
ATT TCC CCC TGT TTC TCA TAT ATA AGT CAT ATT TGG TAG ATA AAA CAG TAT GAT TTG GTT
GGC ATT TTC TTC ATG ATT ATA GGA GCA TCA TTC ATT TGT CTC CAG GGC TAC TTG TGT GAT
ATA GCA TAT ACA TCT ATT GAA GAA AAT AAT CAC TCT CTA GGG GAG GGA GGT AGA AAA GTA
TAT TCT AAA CTT GGG TTT TTG AGT TTG TGG TCT TGT CTT AAC TTT TGT GTT GCT CTA ACT
ACA TGC CAA TAT GTG TTC TCA AGA GTT TTT GTT AAG TAT TGT ATG AAA GTT TAC AGA ATG
AAG GAA GTT CAT CTA CAC TTG AAT CTG TAA GCA AGA TAA CAC ACA AGT GTA CCA AGT GAT
TAT TAA CTT TGT TGT TTT ATA AAT TTG TAT GAA TTT GGA GTA TCT GTT GCC ATT ACT ATA
TAT GTG CAA ATA AAT GTG GCT TAG ACT TGT GAA AAA AAA AAA AAA AAA AA
```
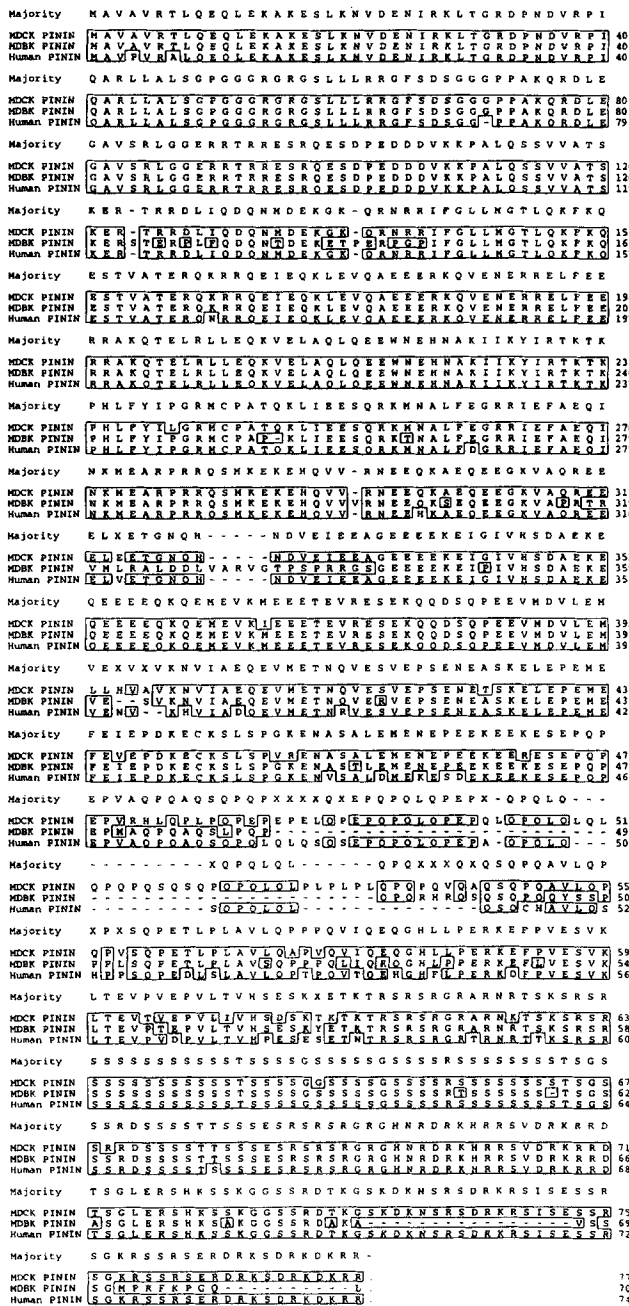
Figure 1. (a) Alignment of the overlapping cDNA clones identified for pinin from MDCK, human placenta, and MDBK. The first two clones identified were po36-5 and po36-9, which represented identical open reading frames of the 3′ end and use of different polyadenylation sites. The sequence derived from the extreme 5′ end of the longer ss13 cDNA clone was used to amplify the 5′ end from cDNA that had been modified by the addition of an anchor sequence. Screening of the human placenta λgt11 library yielded two clones covering most of the coding sequence. The two clones were separated by an internal EcoRI site. The 5′ end of the placental cDNA was also identified by RACE. Overlapping MDBK clones were identified. These clones accounted for the full-length open reading frame and a large 3′ untranslated stretch. (b) The nucleotide and predicted amino acid sequence of MDCK cDNA for pinin. The amino acid sequences obtained from microsequencing fragments that were derived from trypsin digestion of 08L are 26, 28, and 38 (*underlined*), and those derived from V8 digestions are V_1, V_2, and V_3 (*underlined*). The 3′ untranslated domain contains polyadenylation sequences AATAAA (*underlined*). The one used for ss13 is shown at 3759 (*double underline*), and that used in clone po36-5 is shown at residue 2653 (*double underline*). (c) The nucleotide and predicted amino acid sequence of MDBK cDNA.

c

matographic methods, and Western blotting. Pinin was extracted from MDCK cells with 0.4 M KCl and then dialyzed against Tris buffer. The resultant precipitate contained >90% of the total cellular pinin, as judged by Western blotting. The material was made soluble with 4 M urea and separated on a Sephacryl S-400 column. The 08 L immunoreactive fractions were pooled (14 fractions of 0.5 ml of a total of 200 protein containing fractions). The pooled samples were then applied to DEAE and pinin was eluted between 0.17 and 0.23 M NaCl. This yielded a highly enriched pinin fraction. We next separated this fraction on SDS-PAGE and verified the resulting 140-kD band to be pinin by parallel Western blotting. The 140-kD band was excised from nitrocellulose and processed for microsequencing. A total of ~10 μg of pinin that was digested by trypsin and amino acid sequences of peptide products were determined at the Harvard University Microchemistry Laboratory. This method yielded three stretches of amino acid sequences (LLALSGP, 28), (VELAQLQEE-WNEHNAK, 36), and (LTEVTVEPVLIVHSDSK, 38).

The amino acid sequence information obtained by internal microsequencing enabled the preparation of a set of degenerative oligonucleotides for probing a cDNA library. Peptide 36 was selected because of the low degeneracy of nine amino acids at its carboxyl end (EEWNEHNAK). Screening of an oriented MDCK cDNA library constructed in the UNI-ZAP XR vector was used. Three positive clones (po36-4, po36-5, and po36-9) were identified from total of 300,000 phage plaques. Restriction enzyme mapping suggested that po36-4 and po36-5 were nearly identical, and that po36-5 and po36-9 seemed to share an overlapping fragment of 1.5 kb. The total insert length of po36-5 was 2.2 kb, while that of po36-9 was 2.5 kb. Sequencing revealed that the 3' end of po36-9 was different than po36-5, presumably because of the use of an alternate polyadenylation signal. Rescreening of the library with a 1.6-kb EcoRI fragment of po36-5 identified one significantly longer clone, ss13 (Fig. 1 a). This clone was sequenced. The 3' end of ss13 showed complete identity with po36-5 and contained an additional 600 bp at the 5' end. We obtained the remainder of the full-length cDNA by the 5' RACE procedure. Using nested primers complimentary to the sequence near the 5' end of the ss13 clone and the anchor sequence ligated to the end of the reverse transcriptase product, the remaining 5' end was amplified, and the cDNA extending beyond the translation initiation site was revealed (Fig. 1 a).

Sequence of Pinin

The MDCK cDNA contains a single large open reading frame of 2,316 bp ending with a TAA stop codon. The putative 3' untranslated region of 1.5 kb includes an in-frame stop codon 180 bases downstream and out-of-frame stop codons at 18 and 54 bases downstream. The polyadenylation signal AATAAA was found at basepair 3,949, ~20 bases upstream from the poly (A) tail (Levitt et al., 1989). The amino acid sequences of the three tryptic fragments, 28, 36, and 38, were found within the open reading frame defined by ss13 (Fig. 1 b, underlined letters). In addition, the sequences LQPLP, LQLPLPLPL, and LQPQP were found within the open reading frame (Fig. 1 b, underlined).

Figure 2 (amino acid sequence alignment):

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Majority  M A V A V R T L Q E Q L E K A K E S L K N V D E N I R K L T G R D P N D V R P I
MDCK PININ   M A V A V R T L Q E Q L E K A K E S L K N V D E N I R K L T G R D P N D V R P I  40
MDBK PININ   M A V A V R T L Q E Q L E K A K E S L K N V D E N I R K L T G R D P N D V R P I  40
Human PININ  M A V P L V R A L Q E Q L E K A K E S L K N V D E N I R K L T G R D P N D V R P I  40

Majority  Q A R L L A L S G P G G G R G R G S L L L R R G F S D S G G G P P A K Q R D L E
MDCK PININ   Q A R L L A L S G P G G G R G R G S L L L R R G F S D S G G G P P A K Q R D L E  80
MDBK PININ   Q A R L L A L S G P G G G R G R G S L L L R R G F S D S G G G P P A K Q R D L E  80
Human PININ  Q A R L L A L S G P G G G R G R G S L L L R R G F S D S G G G I P P A K Q R D L E  79

Majority  G A V S R L G G E R R T R R E S R Q E S D P E D D D V K K P A L Q S S V V A T S
MDCK PININ   G A V S R L G G E R R T R R E S R Q E S D P E D D D V K K P A L Q S S V V A T S  120
MDBK PININ   G A V S R L G G E R R T R R E S R Q E S D P E D D D V K K P A L Q S S V V A T S  120
Human PININ  G A V S R L G G E R R T R R E S R Q E S D P E D D D V K K P A L Q S S V V A T S  119

Majority  K E R - T R R D L I Q D Q N M D E K G K - Q R N R R I F G L L N G T L Q K F K Q
MDCK PININ   K E R - T R R D L I Q D Q N M D E K G K - Q R N R R I F G L L N G T L Q K F K Q  158
MDBK PININ   K E R S T E R B L P Q D Q N T D E K E T P B R E G P I F G L L N G T L Q K F K Q  160
Human PININ  K E R - T R R D L I Q D Q N M D E K G K - Q R N R R I F G L L N G T L Q K F K Q  157

Majority  E S T V A T E R Q K R R Q E I E Q K L E V Q A E E E R K Q V E N E R R E L F E E
MDCK PININ   E S T V A T E R Q K R R Q E I E Q K L E V Q A E E E R K Q V E N E R R E L F E E  198
MDBK PININ   E S T V A T E R Q K R R Q E I E Q K L E V Q A E E E R K Q V E N E R R E L F E E  200
Human PININ  E S T V A T E R Q N R R Q E I E Q K L E V Q A E E E R K Q V E N E R R E L F E E  197

Majority  R R A K Q T E L R L L E Q K V E L A Q L Q E E W N E H N A K I I K Y I R T K T K
MDCK PININ   R R A K Q T E L R L L E Q K V E L A Q L Q E E W N E H N A K I I K Y I R T K T K  238
MDBK PININ   R R A K Q T E L R L L E Q K V E L A Q L Q E E W N E H N A K I I K Y I R T K T K  240
Human PININ  R R A K Q T E L R L L E Q K V E L A Q L Q E E W N E H N A K I I K Y I R T K T K  237

Majority  P H L F Y I P G R M C P A T Q K L I E E S Q R K M N A L F E G R R I E F A E Q I
MDCK PININ   P H L F Y I L G R M C P A T Q K L I E E S Q R K M N A L F E G R R I E F A E Q I  278
MDBK PININ   P H L F Y I P G R M C P A P - K L I E E S Q R K T N A L F E G R R I E F A E Q I  279
Human PININ  P H L F Y I P G R M C P A T Q K L I E E S Q R K M N A L F D G R R I E F A E Q I  277

Majority  N K M E A R P R R Q S H K E K E H Q V V - R N E E Q K A E Q E E G K V A Q R E E
MDCK PININ   N K M E A R P R R Q S H K E K E H Q V V - R N E E Q K A E Q E E G K V A Q R E E  317
MDBK PININ   N K M E A R P R R Q S H K E K E H Q V V V R N E E Q K G E Q E E G K V A Q R T R  319
Human PININ  N K M E A R P R R Q S H K E K E H Q V V - R N E E H K A E Q E E G K V A Q R E E  316

Majority  E L X E T G N Q H - - - - - N D V E I E E A G E E E K E I G I V K S D A E K E
MDCK PININ   E L E E T G N Q H - - - - N D V E I E E A G E E E K E I G I V K S D A E K E  352
MDBK PININ   V M L R A L D D L V A R V G T P S P R R G S G E E E E K E I D I V H S D A E K E  359
Human PININ  E L D V E T G N Q H - - - - N D V E I E E A G E E E K E I G I V H S D A E K E  351

Majority  Q E E E E Q K Q E M E V K M E E E T E V R E S E K Q Q D S Q P E E V M D V L E H
MDCK PININ   Q E E E E Q K Q E M E V K L E E E T E V R E S E K Q Q D S Q P E E V M D V L E H  392
MDBK PININ   Q E E E E Q K Q E M E V K M E E E T E V R E S E K Q Q D S Q P E E V M D V L E H  399
Human PININ  Q E E E E Q K Q E M E V K M E E E T E V R E S E K Q Q D S Q P E E V M D V L E H  391

Majority  V E X V X V K N V I A E Q E V M E T N Q V E S V E P S E N E A S K E L E P E M E
MDCK PININ   L L H V A V K N V I A E Q E V M E T N Q V E S V E P S E N E L T S K E L E P E M E  432
MDBK PININ   V E - - S V K N V I A E Q E V M E T N Q V E R V E P S E N E A S K E L E P E M E  437
Human PININ  V E N V - - L H L V I A D L Q E V M E T N R V E S V E P S E N E A S K E L E P E M E  429

Majority  F E I E P D K E C K S L S P G K E N A S A L E M E N E P E E K E E K E S E P Q P
MDCK PININ   F E V E P D K E C K S L S P V R E N A S A L E M E N E P E E K E R E S E P Q P  471
MDBK PININ   F E I E P D K E C K S L S P G K E N A S T L E M E N E P E E K E E K E S E P Q P  477
Human PININ  F E I E P D K E C K S L S P G K E N V L S A L D M E F K L S D E K E E K E S E P Q P  469

Majority  E P V A Q P Q A Q S Q P Q P X X X X Q X E P Q P Q L Q P E P X - Q P Q L Q - - -
MDCK PININ   E P V R H L Q L P L P Q P L P E P E L Q P E P Q P Q L Q P Q P Q L Q L Q L  512
MDBK PININ   E P H A Q P Q A Q S L P Q P - - - - - - - - - - - - - - - - - - - - - - -  492
Human PININ  E P V A Q P Q A Q S Q P Q L Q L Q S Q S E P Q P Q L Q P E P A - Q P Q L Q - - -  506

Majority  - - - - - - - - - X Q P Q L Q L - - - - - Q P Q X X X Q X Q S Q P Q A V L Q P
MDCK PININ   Q P Q P Q S Q S Q P Q P Q L Q L P L P L P L Q P Q P Q V Q A Q S Q P Q A V L Q P  552
MDBK PININ   - - - - - - - - - - - - - - - - - - - - - - - Q P Q R H R Q S Q S Q P Q Q Y S S P  509
Human PININ  - - - - - - - - - S Q P Q L Q L - - - - - - - - - - - - Q S Q C H A V L Q S  522

Majority  X P X S Q P E T L P L A V L Q P P P Q V I Q E Q G H L L P E R K E F P V E S V K
MDCK PININ   Q P V S Q P E T L P L A V L Q A P V Q V I Q E Q G H L L P E R K E F P V E S V K  592
MDBK PININ   P P L S Q P E T L P L A V S Q P P F Q L I Q R Q G H L P P E R K E F L V E S V K  549
Human PININ  H P P L S Q P E D L L S L A V L Q P T Q V T Q E H L G H F L P E R K N D P E S V K  562

Majority  L T E V P V E P V L T V H S E S K X E T K T R S R S R G R A R N R T S K S R S R
MDCK PININ   L T E V T V E P V L I V H S D S K T K T K T R S R S R G R A R M K T S K S R S R  632
MDBK PININ   L T E V P T E P V L T V H S E S K Y E T K T R S R S R G R A R N R T S K S R S R  589
Human PININ  L T E V P V D L P V L T V H P L S E S I E T M T R S R S R G N T R N R T T K S R S R  602

Majority  S S S S S S S S S S S T S S S S G S S S S G S S S S R S S S S S S S S T S G S
MDCK PININ   S S S S S S S S S S S T S S S G Q S S S S G S S S S R S S S S S S S S T S G S  672
MDBK PININ   S S S S S S S S S S S T S S S S G S S S S G S S S S R S S S S S S S S T S G S  628
Human PININ  S S S S S S S S S S T S S S S G S S S S G S S S S R S S S S S S S S T S G S  642

Majority  S S R D S S S S T T S S S E S R S R S R G R G H N R D R K H R R S V D R K R R D
MDCK PININ   S R R D S S S S T T S S S E S R S R S R G R G H N R D R K H R R S V D R K R R D  712
MDBK PININ   S S R D S S S S T T S S S E S R S R S R G R G H N R D R K H R R S V D R K R R D  668
Human PININ  S S R D S S S S T S S S E S R S R S R G R G H N R D R K H R R S V D R K R R D  682

Majority  T S G L E R S H K S S K G G S R D T K G S K D K N S R S D R K R S I S E S S R
MDCK PININ   T S G L E R S H K S S K G G S R D T K G S K D K N S R S D R K R S I S E S S R  752
MDBK PININ   A S G L E R S H K S A K G G S S R D A K A - - - - - - - - - - - - - V S G  692
Human PININ  T S G L E R S H K S S K G G S R D T K G S K D K N S R S D R K R S I S E S S R  722

Majority  S G K R S S R S E R D R K S D R K D K R R -
MDCK PININ   S G K R S S R S E R D R K S D R K D K R R -  774
MDBK PININ   S G M P R F K P C Q - - - - - - - - - L -  704
Human PININ  S G K R S S R S E R D R K S D R K D K R R -  744
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Figure 2. Alignment of the predicted amino acid sequence for MDCK pinin, MDBK pinin, and human placental pinin. Residues that are boxed match the consensus (majority) exactly. Note that the amino terminus and the polyserine domains are highly conserved for these species, whereas there is significant variation in the sequence of the QLQP domain.

These sequences were previously obtained from the microsequencing 30–31-kD, 08L-immunoreactive *Staphylococcus aureus* V8 protease products of pinin. This cluster of partial V8 protease digestion fragments most likely represented the carboxyl end of pinin cleaved by the V8 enzyme at glutamine residues within the QLQP domain.

The full-length cDNA identified from MDCK and humans have an extremely short 5' untranslated region of 54 bp. From the size of pinin mRNA determined from Northern blots (longest being 4.1 kb; see Fig. 4) and primer extension analyses (data not shown), it appears that we are missing 350–400 bp of the 5' untranslated region of the MDCK gene. Screening of human placenta and MDBK libraries provided clones covering most of the open reading frame. The extreme 5' end of the human placenta cDNA was identified by 5' RACE. The human and bovine sequences exhibited extensive similarity to the MDCK pinin sequence (Figs. 1 c and 2). MDBK cDNA included a large 5' untranslated region of 1.7 kb (Fig. 1 c). The first ATG in the MDCK cDNA was identified as the start codon by similarity of the region containing it to the Kozak consensus sequence for the initiation of translation (Kozak, 1991). The methionine codon is within a sequence in which the critical −3 position is occupied by a purine (A) and the critical +4 position is occupied by (G). This site of translation has been identified to be the same in cDNAs from dog (MDCK), cow (MDBK), mouse (embryo), and human (placenta).

Expression of cDNA in Escherichia coli and Production of Antibodies

Additional evidence that this cDNA was indeed coding for pinin was obtained from the expression of cDNA-encoded proteins and immunoblotting, and finally, the production of new antibodies directed against fusion proteins. Attempts to express full-length ss13 or po36-5 in *E. coli* were not successful. The recombinant proteins were unstable, presumably because of the highly charged regions of pinin. We therefore expressed smaller portions of pinin. PCR was used to generate partial cDNAs coding for 10–15-kD polypeptide stretches of pinin. Products were ligated to the pGEX-1 expression vector. We expressed five stretches of pinin. Western blots of recombinant proteins demonstrated that peptides containing the extreme carboxyl terminus were immunostained with 08L antibody. This location was consistent with our earlier observation that V8 protease digestion of pinin yielded multiple immunoreactive products of ∼30 kD, with QP-containing sequences at their amino ends.

An expressed peptide, which contained the QPQL domain and was not immunoreactive to the 08L antibody, was selected for use as an immunogen to generate new antisera against the cDNA-encoded protein. Antisera from rabbits inoculated with fusion protein 3 immunostained the 140-kD pinin on Western blots of MDCK extracts (Fig. 3 a). Immunofluorescence microscopy revealed that the new antibody (designated m3-AG) immunostained the lateral cell surfaces of epithelial cells consistent with the 08 L staining pattern (Fig. 3 b). Taken together, these data confirm that the po36-5 and ss13 cDNAs, identified here, indeed code for the 140-kD 08L antigen localized to the IF-desmosomal complex.

Tissue Expression of Pinin

Northern blots probed with either the 1.6-kb EcoRI fragment or the 280-bp EcoRI-AccI fragment of ss13 (which does not contain the coding region for the QPQL or polyserine domains) revealed that there were at least three mRNA species that bind under high stringency (Fig. 4).
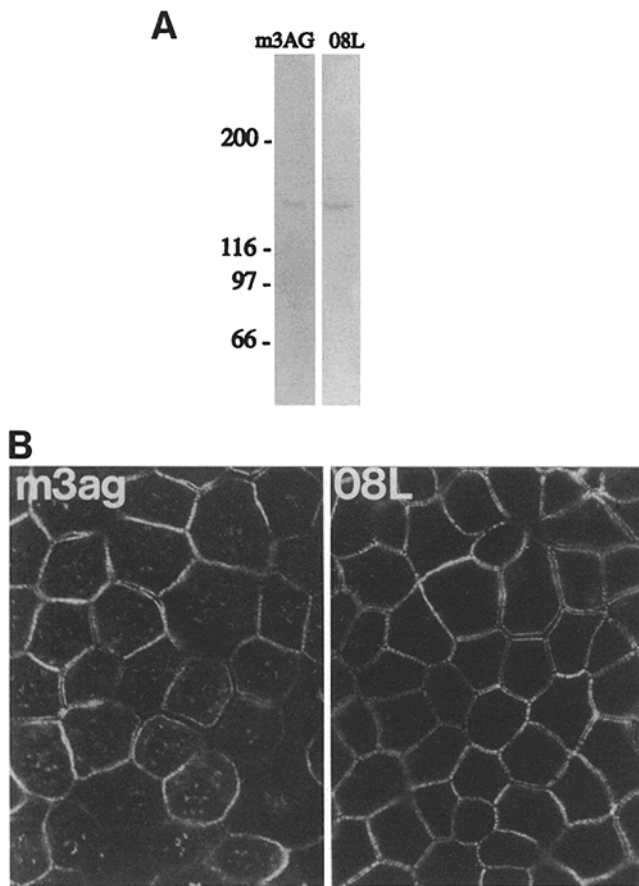
Figure 3. (a) Polyclonal antiserum directed against fusion protein 3 was used to immunostain Western blots of MDCK preparations. Antibody m3AG immunostained the same 140-kD protein as did the 08L antibody. (b) The antibody was used to immunostain confluent cultures of MDCK cells. The antisera immunostained the lateral epithelial boundaries. This was seen as linear staining of the cells, often producing parallel lines between neighboring cells. This pattern was consistent with the staining pattern seen with the 08L antibody. The 3AG also immunostained aggregates within the cytosol that did not label with 08L. Whether this cytosolic staining is indeed pinin or a related protein is currently under examination.

RNA from MDCK cells contained a 4.1-kb message for pinin, while RNA from the stratified epithelium of the cornea contained a 3.7-kb message. When the multiple human tissue Northern blots were probed with pinin cDNA, a tissue-specific heterogeneity in message sizes was revealed (Fig. 4 b). Whereas the placenta, lung, liver, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and heart (intercalated disks) all contain desmosomes and therefore would be expected to contain pinin mRNA, the presence of message for pinin in brain and skeletal muscle is somewhat puzzling.

The chicken brain, when immunostained with 08L antibody, demonstrated obvious desmosomal staining of only meningeal layers, while the nervous tissue presented a homogeneous, muted positive staining. Certain axonal tracts seemed to present a more distinct signal. Immunostaining of cultured nerve cells, however, demonstrated a distinct pattern of pinin immunoreactivity (Fig. 5, a–c). Pinin immunoreactivity was seen along borders of nerve cell bod-

ies as well as along axons. While these locations stained intensely with neurofilament antibodies, they were negative for the desmosomal component desmoplakin. These data suggest that the distribution of pinin and/or pinin isoforms may, in fact, be more widespread than just desmosome associated.

While the presence of the larger mRNA may be consistent desmosome-containing tissues, the ubiquitous, smaller mRNA may code for a pinin-related protein and not the desmosome-associated pinin. We see no 08L immunostaining of blood cells that do not contain desmosomes, yet the smaller mRNA is present in leukocytes. This leukocyte mRNA is consistent with the report of Bellavite et al. (1990), who have identified a partial cDNA that may code for a neutrophil phosphoprotein that exhibits a great similarity to pinin's carboxyl end. The possibility of these tissues containing a different but related protein to pinin is currently under examination, as is the possibility of alternative splicing of pinin mRNA similar to that seen in desmoplakin I and II (Angst et al., 1990). While genomic human Southern blots probed with pinin cDNA under high stringency conditions revealed hybridization to two bands after EcoRI and HindIII digestion, one distinct hybridizing band was seen after digestion with PstI (Fig. 6). These data are consistent with the existence of one pinin gene. However, additional experiments are required to definitively resolve whether there are two pinin-related genes, alternative splicing, or both.

Expression of Pinin cDNA in Cultured Cells

The predicted molecular mass of pinin is 88.1 kD, with an isoelectric point of 6.5, although this protein migrates at 140 kD on SDS gels (Ouyang and Sugrue, 1992). Expression of the full-length clones in mammalian cells (monkey COS-7) resulted in the production of a 140-kD band on Western blots (Fig. 7). The 140-kD protein was identified in whole-cell lysates of COS-7 cells that had been transfected with a full-length insert ligated into pCDNA$_3$. Nontransfected COS-7 cells showed no detectable levels of pinin (Fig. 7). Expression of myc epitope–tagged pinin resulted in the production of a 140-kD myc-immunoreactive band on Western blots (data not shown). While posttranslational modification may contribute to the altered SDS-PAGE mobility, we do not feel it alone accounts for such a large change. Indeed, phosphatase treatment of cell lysates only minimally shifts the apparent molecular weight. It is also possible that pinin is cross-linked to another component. However, we have not seen any evidence for cross-linking in pulse chase experiments, nor have we observed a shift in the apparent molecular mass after reduction and alkylation. Mobility on SDS-PAGE appearing slower than predicted from sequence is a fairly common anomaly (Himmler et al., 1989; Field, 1995). Many proteins exhibiting this phenomenon evidently do not fold to form typical globular proteins. Some of our GST fusion proteins (fusion proteins containing fragments 3 and 12) also migrated slower (larger; 50 and 70 kD) than the predicted molecular mass (39 and 53 kD). Interestingly, these fragments both contain the QP domain, which may form a polypeptide stretch containing a series of fixed kinks in the chain.
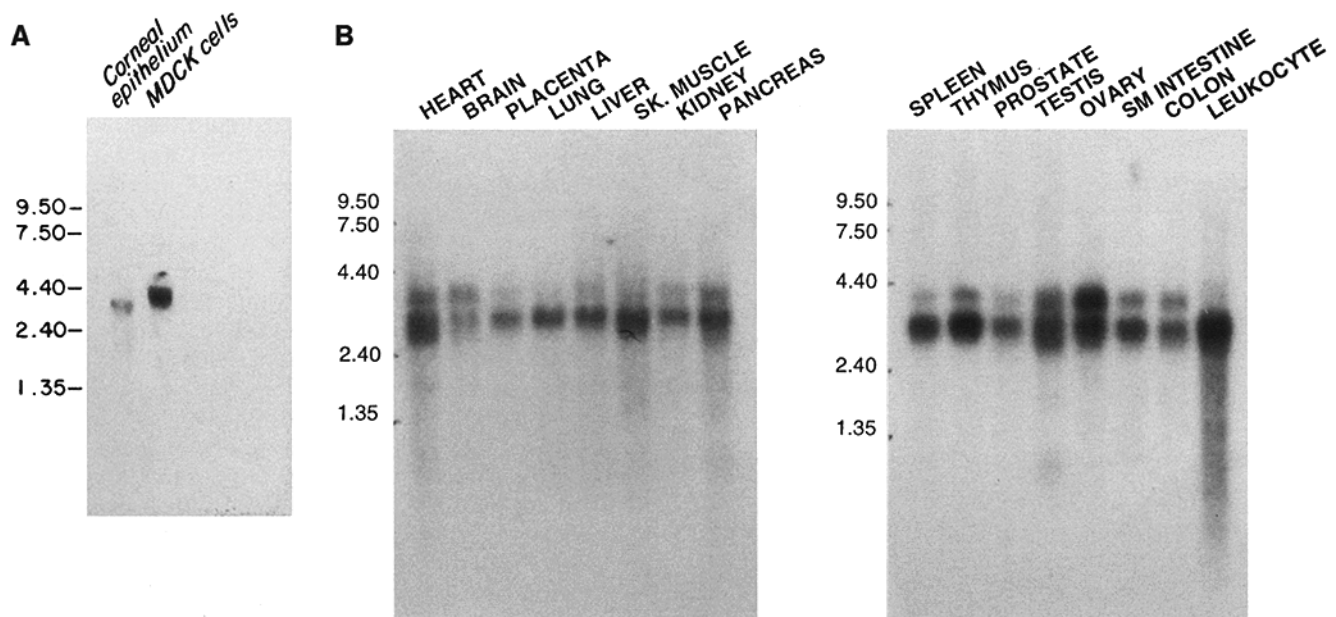
Figure 4. Northern blot analysis of tissue expression of 08L messages revealed that there exists at least three mRNA species that bind 08L DNA under high stringency. (*a*) Probing with the 1.5-kb EcoRI fragment showed that RNA from chick corneal epithelia contained a 3.7-kb message, whereas MDCK cells contained 4.1-kb message. (*b*) Various human tissue mRNA are probed with the EcoRI 1.6-kb fragment of po36-5 (*left panel*) and a 5' fragment containing the EcoRI–AccI 280 bp (*right panel*). Note the appearance of three mRNA sizes in the range of 3.2–4.1 kb, with a tissue-specific variation of expression. The 08L-positive tissues, such as the brain, placenta, liver, kidney, and pancreas contain the 4.1- and the 3.7-kb messages, whereas the heart, lung, and muscle contain predominantly a 3.2-kb message.

Transfection of pinin into chicken embryonic fibroblasts resulted in the cytosolic accumulation of pinin immunoreactivity, but expressing cells demonstrated little change in their morphology. We next examined the distribution of expressed pinin in the context of an epithelial cell. We selected human embryonic kidney–derived 293 cells because they demonstrated expression of epithelial proteins such as cadherins and desmoplakin with little or no pinin expression (Fig. 8, *a* and *a'*). Examination of the 293 cells that had received the full-length pinin revealed expressed pinin along the lateral borders of 293 cells in close contact. The 293 cells that were transfected with pinin cDNA and immunostained for pinin and desmoplakin revealed that expressed pinin was found in association with desmoplakin (Fig. 8, *b* and *b'–d* and *d'*). While the cells expressing the cDNA also exhibited cytosolic accumulations of pinin, there were clearly areas of colocalization, especially at cell–cell contact sites. However, we did not observe significant amounts of pinin assembled at the cell periphery in the absence of desmoplakin.

The expression of pinin in 293 cells leads to a striking change in the cell/tissue morphology. Non–pinin-expressing 293 cells are often seen as spindle shaped, which exhibit limited cell–cell interactions even at very high cell densities. 293 cells transfected with pinin cDNA and then selected with G418, however, exhibited extensive cell–cell contacts and grew in culture as islands (Fig. 9). We have isolated four independent clones of cells expressing pinin. After nine passages of these cells, the epithelial phenotype and growth characteristics of pinin-expressing cells is consistent. EM of these clones revealed that the entire array of epithelial cell junctions is enhanced (Figs. 10 and 11).

While desmoplakin immunostaining of cultures of nontransfected 293 cells revealed moderate staining (Fig. 8 *a'*), morphologically recognizable desmosomes were quite rare in these cells, and those that were found appeared immature and somewhat delicate (Fig. 11 *b*). Examination of pinin-expressing 293 cells revealed fairly well-formed intercellular junctional specializations with numerous small desmosomes (Fig. 11, *c–f*). The desmosomes in the transfected cells, while small, exhibited well-formed plaques and numerous associated intermediate size filaments (Fig. 11, *c–f*). Immunostaining pinin-transfected cells with antibody against the tight junction component ZO1 (Stevenson et al., 1986) revealed deposition of ZO1 along lateral cell borders, while the untransfected 293 cells showed little ZO1 staining and no zonula staining pattern (Fig. 12). While double immunostaining for pinin and ZO1 in transfected cells demonstrated some overlap, the pinin immunostaining was more extensive and showed more interruptions than that for ZO1.

Discussion

We have previously demonstrated that young desmosomes containing desmoglein, desmoplakin, plakoglobin, and associated IF do not exhibit immunoreactivity for pinin. On the other hand, more mature, well-formed, and better organized desmosomes do contain pinin. Therefore, we considered pinin to be a novel protein that is nonessential for desmosomes per se, but perhaps important in the stabilization and organization of the desmosome-IF complex. We set out to gain more insight into the possible functions of pinin and its role in desmosome regulation.
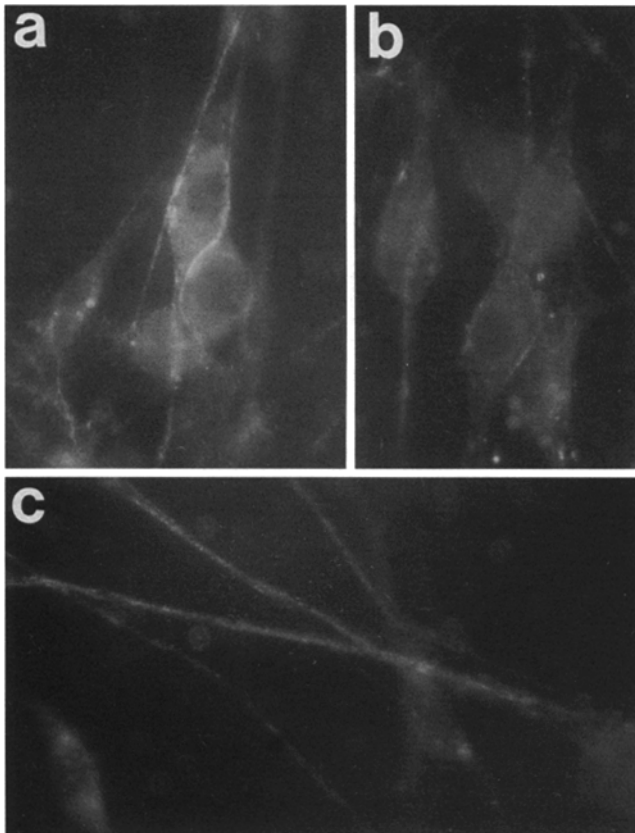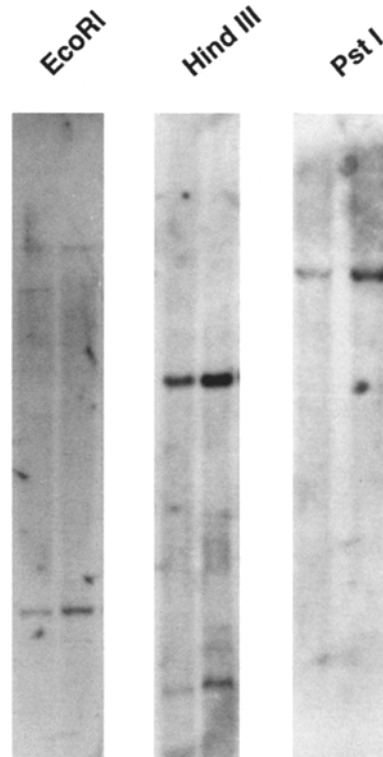
Figure 5. Chick dorsal root ganglia cells were isolated and plated on collagen and were immunostained for pinin. Note that pinin immunoreactivity can be seen along neurites and cell body boundaries (*a–c*). The staining associated to the cell body occasionally appeared to be within neurites wrapped around the cell body (*a*) and within the cell body proper distributed along the cell–cell boundary (*b*). The pinin immunoreactivity appeared punctate along the axonal processes and extended for the length of the axon (*c*).

Figure 6. Genomic DNA was isolated from the peripheral blood and restricted with EcoRI, HindIII, or PstI, and was probed with the 1.5-kb EcoRI fragment of the human pinin clone sshp. While both EcoRI and HindIII digests showed two hybridizing bands, the PstI digest clearly showed only one band.

An essential step in these analyses was to clone the cDNA for pinin.

Pinin Sequence

The predicted amino acid sequence of pinin provided little information regarding the function of the protein. It, however, contains many recognizable domains and motifs that may provide us with some suggestions and directions to pursue. There are two stretches of sequences typical of IFs and IF-associated proteins. Near the amino end, there is a series of four heptad repeats (Fig. 8). These repeats characterize the sequences that form coiled-coil rod domains in α-fibrous proteins such as IFs (Conway and Parry, 1990). The shortest peptides still exhibiting stable coiled-coil structures are four to five heptads long (O'Shea et al., 1989; Conway and Parry, 1990; Oas et al., 1990). Two glycine loop sequence segments are found on the carboxyl side of the heptad repeat region. These tandem quasi-repeat peptides, which are rich in glycine, are widespread in at least three families of proteins, IF proteins, loicrins (envelope components of terminally differentiated epithelial cells; Hohl et al., 1991), and single-stranded RNA-


Figure 7. Western blot of expressed pinin demonstrates the presence of a 140-kD immunoreactive band. Whole-cell lysates were prepared from MDCK, COS-7, and two independent clones of COS-7 cells transfected with pCDNA3 plasmid containing the full-length pinin coding sequence. Note that the 140-kD band, while not visible in parental COS-7, is present in lysates from MDCK and COS-7 transfected with full-length, pinin-containing plasmids. Immunoreactive bands of the 110–120-kD range are present in extracts from transfected cells. These bands presumably represent breakdown products of the 140-kD polypeptide. Similar size, although weaker bands, are seen in extracts from MDCK cells (Ouyang and Sugrue, 1992).
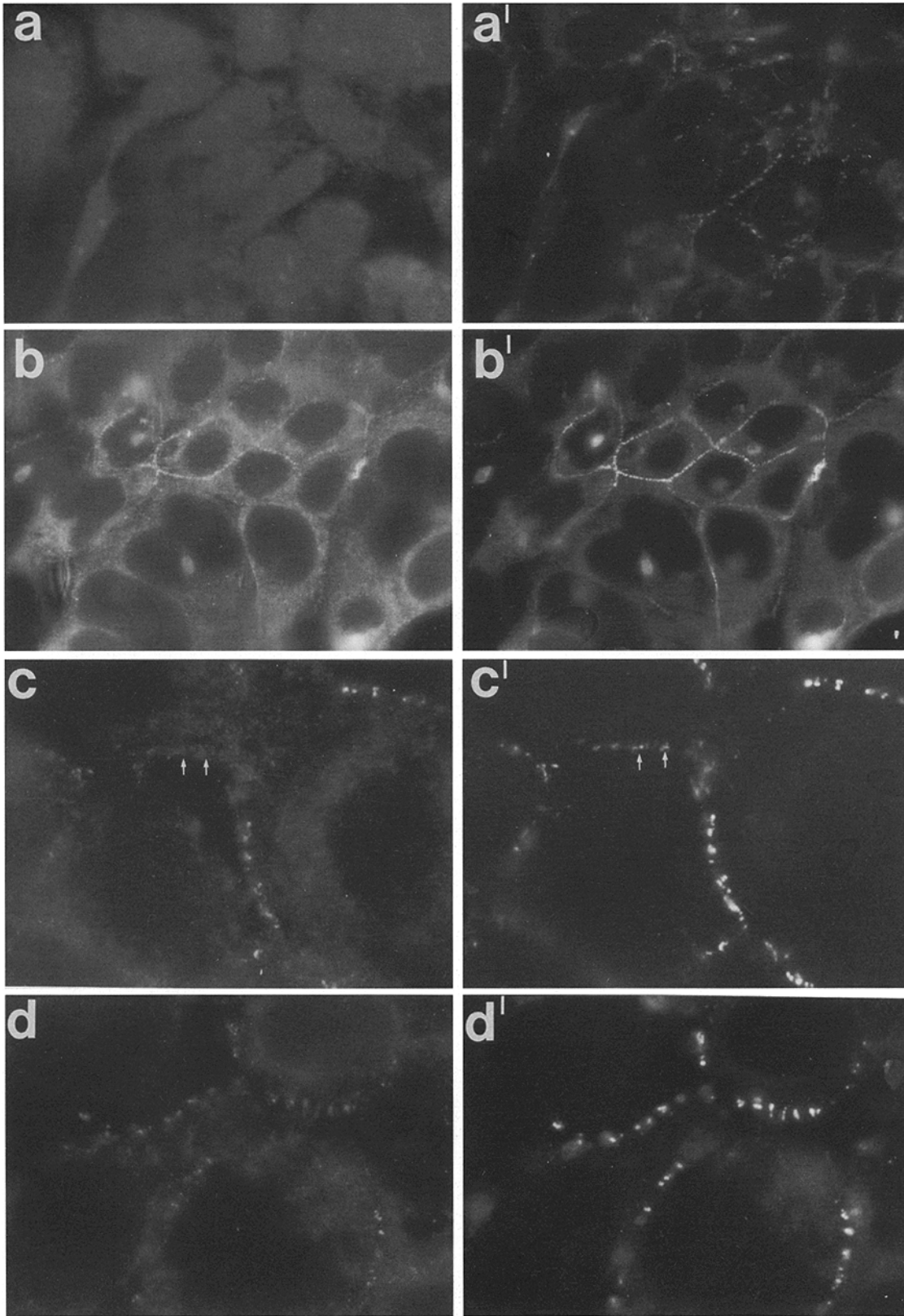
Figure 8. 293 cells transfected with control pCDNA3 plasmid (*a* and *a'*) or pCDNA3 containing the full-length pinin cDNA (*b–d* and *b'–d'*) were immunostained with an antibody directed against pinin (*a–d*) and desmoplakin (*a'–d'*). The untransfected 293 cells, as well as 293 cells that received control DNA, exhibited little or no immunostaining for pinin. However, these cells did show some punctate staining for desmoplakin. The pinin-transfected cells exhibited a high cytoplasmic staining for pinin (*b*). Presumably, this represents an overexpression of pinin. In higher density cultures, pinin can be seen as spots all along the lateral membrane. Pinin cDNA–transfected cells revealed that pinin was associated to desmoplakin. Note that the expressed pinin is located at or near desmoplakin-reactive areas, and pinin is not localized to the cell periphery without desmoplakin. Desmoplakin, however, was seen along cell borders without corresponding to significant pinin staining (*c* and *c'*, *arrows*). Furthermore, pinin-expressing 293 cells demonstrate intracellular accumulations of pinin that do not correspond to desmoplakin-positive areas.
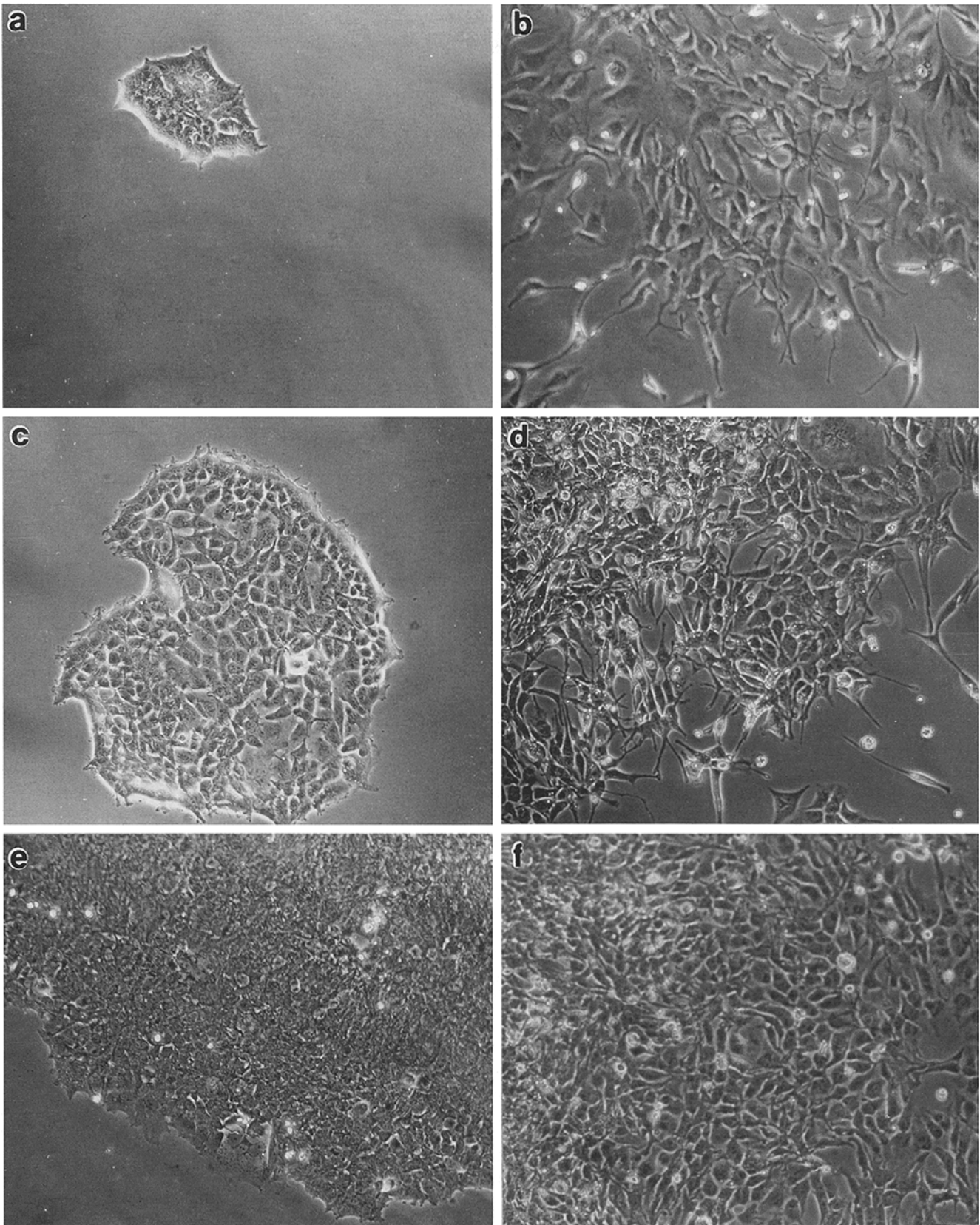
Figure 9. Human embryonic kidney cells (293 cells) transfected with pinin cDNA exhibited an alteration in their phenotype. The 293 cells that received pinin cDNA remained in an epithelial island (*a*, *c*, and *e*), whereas those receiving the control plasmid (*b*, *d*, and *f*) exhibited a spindle shape with numerous cells migrating away from the more dense cell clusters. The difference in culture characteristics was not caused by culture density; similar differences were seen at all densities.
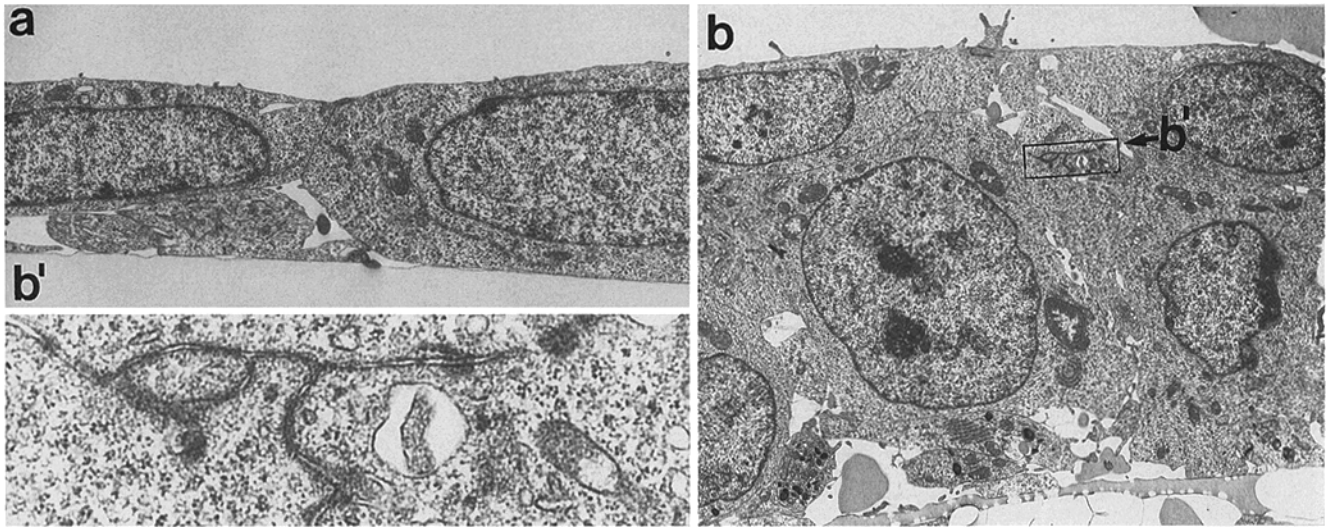
Figure 10. EM of pinin-transfected 293 cells showed enhanced cell–cell adhesion with more extensive cell junctions. Untransfected 293 cells grown at a high cell density demonstrated cell adhesion with limited cell junctions (*a*). Even at high densities, the 293 cells retained a low profile. Pinin-transfected 293 cells demonstrated cell junctional complexes and a more typical epithelial polarity (*b*). Lateral cell surfaces displayed numerous junctions, including desmosomes (*b'*, as outlined in *b*).

binding proteins (Steinert et al., 1991). Glycine loops are expected to be highly flexible, and may participate in interactions with neighboring glycine loops on the same or adjacent proteins. They have been postulated to form the basis for adaptable intracytoskeleton interactions (Steinert et al., 1991). Residues 635–700 comprise a serine-rich domain. Serines comprise 48 of the 65 residues. The polyserine domain may be highly flexible and may represent a "hot spot" for the phosphorylation of pinin. The deduced sequence of pinin contains motifs recognized by Ca$^+$/calmodulin protein kinases (RXXS), c-AMP–dependent kinases (XRRXSX), c-GMP–dependent protein kinase (XSRX), protein kinase C (XRXXSX), and casein kinase 2 (XSXXEX; Kemp and Pearson, 1990). These potential phosphorylation sites are clustered at two locations: (*a*) around the polyserine domain; and (*b*) near the amino-terminal end of the negatively charged, glutamic acid–rich domain. We have previously shown that pinin exhibits multiple isoforms on two-dimensional gel analyses because of phosphorylation.

A comparison of the cDNA and protein sequence of pinin with other sequences available from databases (GenBank/EMBL/DDBJ, Swiss Prot) revealed a striking homology of the 3' end of pinin cDNA to a cDNA identified from a pig neutrophil expression library. This cDNA was identified with a polyclonal antibody directed against a 32-kD phosphoprotein that was involved in a phosphorylation cascade of neutrophils (Bellavite et al., 1990). Within a 279–amino acid stretch of pinin, 196 amino acids were identical to those found in the 32 kD neutrophil phosphoprotein. At this time, it is not clear as to whether the cDNA identified from the pig neutrophil codes for the 32-kD phosphoprotein or a larger neutrophil protein sharing an antigenic site. While we have not noted any immunostaining of blood cells with antibodies directed against pinin, cDNA probes derived from the 5' end of pinin cDNA recognize a 3.4-kb band in Northern blots of peripheral blood leukocytes. These data may indicate that pinin and

the neutrophil protein are members of a protein family involved in phosphorylation events that take place at the cytoskeleton–membrane interface.

Other than the pig neutrophil cDNA, no significant sequence homologies were detected on homology searches. However, there was some weak homology of the glutamic acid–rich domain to proteins such as trichohyalin, caldesmon, and myosin. The significance of these small homologous stretches is not yet evident. When the cDNA and protein sequence of pinin was directly compared to desmosomal-associated proteins such as desmoplakin, desmocollins, plakoglobin, and plectin (with the exception of the heptad repeats), few if any other significant homologies were observed. There were, however, certain regions that exhibited the β-turn and charge characteristics of the 13-residue repeat found in filagrin, another IF-associated protein (Rothnagel et al., 1987; Rothnagel and Steinert, 1990; Mack, 1993).

Possible Functions of Pinin

Clearly, pinin is predominantly found associated to desmosomes. Nevertheless, we have shown that it is also present within cultured nerve cells, and in fact, Northern blots suggest that it may be expressed at significant levels in the brain. While we do not yet have detailed information as to its distribution in nerve cells, it is surely not associated to desmosomes. Therefore, we must keep in mind that pinin may have a more general role within epithelial cells than that at the desmosome.

While expressing pinin cDNA in fibroblasts yielded little information, expressing it in transformed epithelial cells (human 293 cells) resulted in a dramatic change in cell/tissue architecture. The recipient cells exhibited an increased cell–cell adhesion and an enhanced epithelial cell polarity. We suggest that this increased adhesion may be caused by the stabilization of the desmosomal adhesion
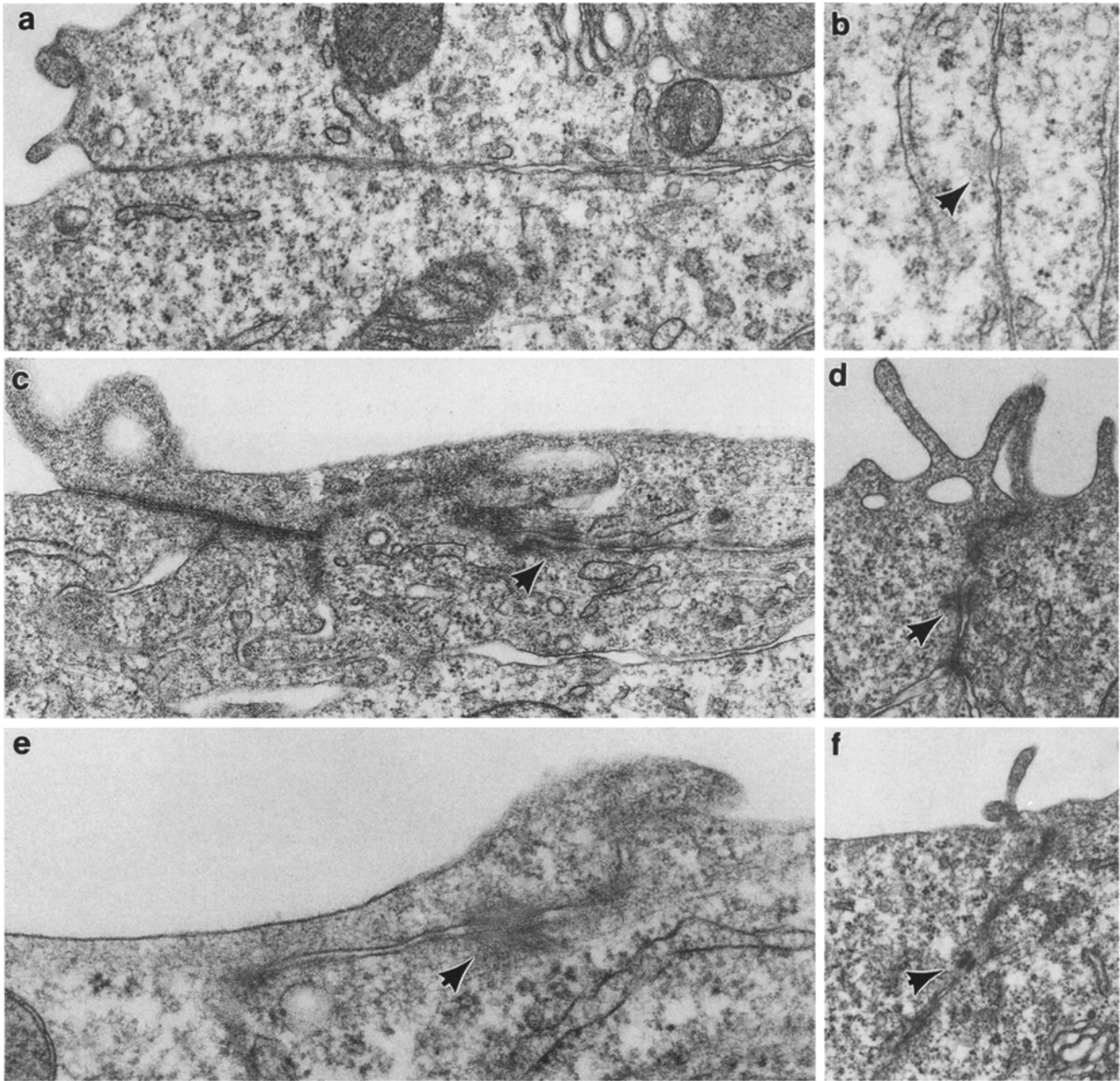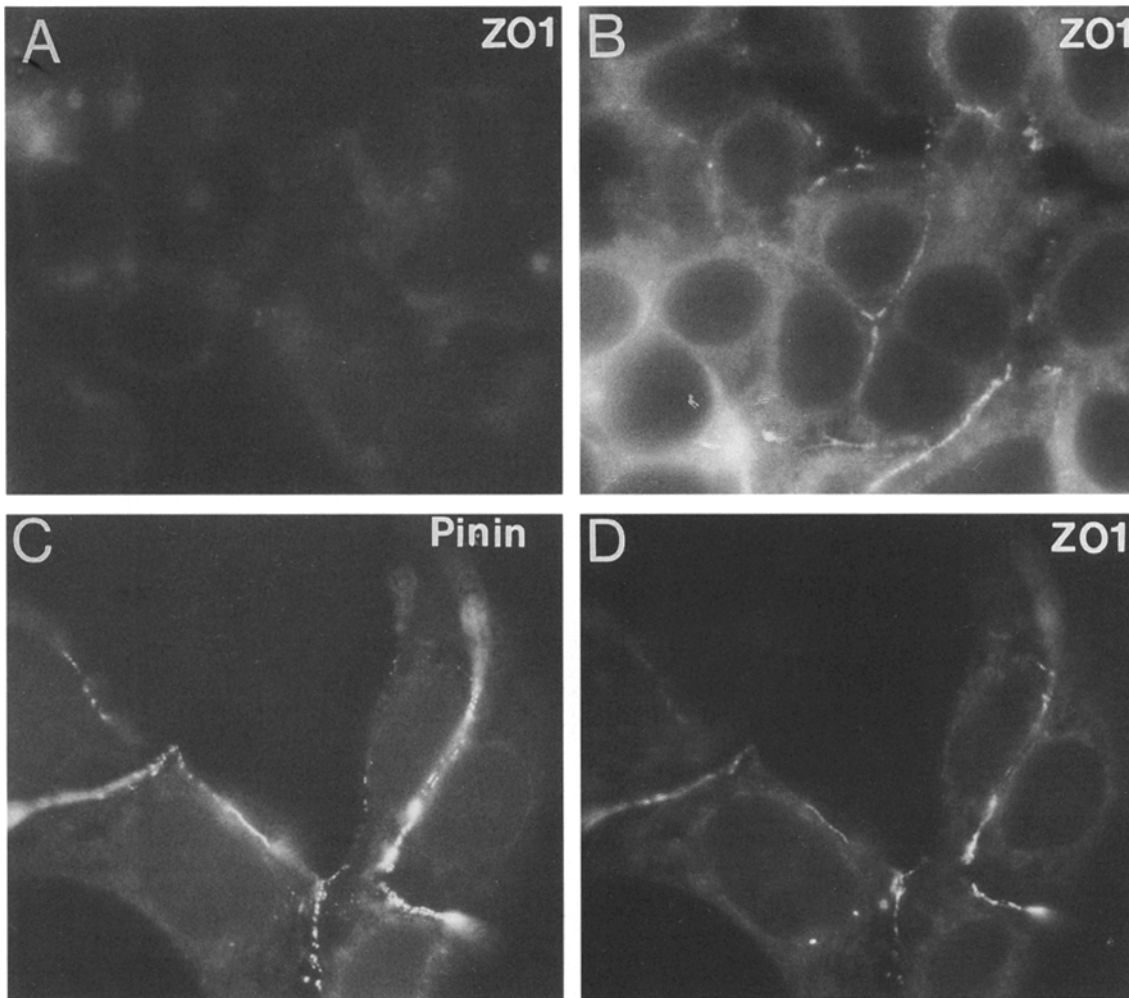
Figure 11. EM of pinin-transfected 293 cells revealed that the pinin cDNA–transfected cells contained a more significant cell–cell junction complex. Examination of nontransfected and control plasmid–transfected cells (*a* and *b*) revealed limited adhesion specializations along cell–cell contact surfaces. Desmosomes were seen only occasionally and they appeared small and immature (*b*). The pinin-transfected cells, however, demonstrated a more extensive array of junctions, including desmosomes (*c–f, arrowheads*), well-formed adherens junctions, and what appeared to be forming tight junctions.

complexes by the expressed pinin. Immunofluorescence of pinin cDNA–transfected cells demonstrated that pinin was produced and accumulated at cell–cell contact boundaries when put in an epithelial context. Double immunofluorescence studies with pinin and desmoplakin antibodies revealed that, while pinin was observed in numerous cytosolic aggregates, it was only found along cell contact boundaries that also contained desmoplakin. Interestingly, the overexpression of pinin did not seem to result in a general accumulation along the cytosolic side of the plasma membrane in fibroblasts or other nonepithelial cells. The data

suggest that pinin itself does not nucleate the desmosomal junction, but rather, it assembles to a preexisting accumulation of desmoplakin at the cell periphery. Taken together with our previous results on the assembly of pinin to the desmosome, we suggest that pinin may function by assembling to a formed or forming desmosome, thus stabilizing the desmosomal-IF complex.

The correlation of pinin expression with the increased deposition of ZO1 is suggestive that the enhanced epithelial adhesion is affording the epithelial cells the opportunity to stabilize other junctional specializations such as the

Figure 12. Examination of pinin-transfected cells by immunostaining with tight junction–associated protein ZO1 revealed increased deposition of ZO1 along lateral epithelial borders. Untransfected 293 cells grown at high cell densities showed little immunostaining for ZO1 (*A*), whereas pinin-transfected 293 cells showed ZO1 deposits along lateral cell borders (*B*). Double immunostaining of pinin-transfected cells for pinin (*C*) and ZO1 (*D*) revealed linear ZO1 staining near the more extensive punctate staining for pinin.

tight and adherens junctions. ZO1 has been localized to the zonula adherens and to the tight junction (Itoh et al., 1993); therefore, ZO1 in the transformed cells could be located at either or both of the junctions. EM revealed an increase in both tight and adherens junctions along with the increase in desmosomes. While pinin does not overlap with ZO1 in epithelial tissues in vivo (Ouyang and Sugrue, 1992), it will be of great importance to determine whether or not pinin interacts with ZO1 or other nondesmosomal junctional components in transfectant cells.

The serine block, flanked by numerous kinase recognition motif sites, suggests that at least the carboxyl portion of pinin may serve as a substrate for serine/threonine kinases. It has been postulated that phosphorylation may play an important role in cell–cell adhesion (Tsukita et al., 1991; Volberg et al., 1991, 1992, 1994; Takeichi et al., 1992; Stappenbeck et al., 1994), as well as IF and IF-associated protein assembly and function (Nigg et al., 1986; Sihag et al., 1988; Shea et al., 1990; Foisner et al., 1991; Nixon and Sihag, 1991; Peter et al., 1991; Hennekes et al., 1993). In addition, other desmosomal components, such as the

desmoplakins and desmocollins, have been demonstrated to be phosphorylated on serines (Stappenbeck et al., 1994). Citi and co-workers have showed that serine phosphorylation by protein kinase C may be a key step in desmosome disassembly (Citi, 1992; Citi et al., 1994; Denisenko et al., 1994). In desmosome assembly experiments, we have shown that pinin is recruited to an existing desmosome. If pinin is involved in the maintenance of desmosomal stability, then it may be reasonable to speculate that phosphorylation of pinin at key sites may induce a conformational change in the molecule, thus weakening its association to the desmosomal complex and thereby lowering the stability of the desmosome. It will be of great interest to determine the role of specific phosphorylation of pinin domains in the assembly and disassembly of desmosomes and stable epithelial adhesion.

Here, we have presented data regarding the characterization of a novel desmosome-associated molecule, pinin. The identification of the cDNA for pinin as genuine was supported by the predicted amino acids that were deduced from the cDNA sequences containing all three tryptic

fragments derived from the purified protein. In addition, the original 08L mAb reacted with fusion proteins that were expressed in vitro. Moreover, antiserum that was collected from animals injected with this recombinant protein identified the 140-kD pinin on Western blots and immunostained the lateral epithelial surfaces. Expression of the full-length cDNA clones in 293 cells demonstrated that pinin was produced and assembled along the lateral cell surface, where it was localized near desmoplakin. Furthermore, cells receiving pinin cDNA exhibited enhanced cell–cell adhesion. We believe that investigation into the function(s) of pinin and related proteins in cell adhesion and IF organization will contribute significantly to our current knowledge of epithelial cell–cell adhesion.

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