Dissection of Protein Linkage between Keratins and Pinin, a Protein with Dual Location at Desmosome-Intermediate Filament Complex and in the Nucleus*

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Pinin is a cell adhesion-associated and nuclear protein that has been shown to localize in the vicinity of intermediate filament (IF) convergence upon the cytoplasmic face of the desmosomal plaque as well as in the nucleus. The localization of pinin to the desmosomes has been correlated with the reinforcement of intercellular adhesion and increased IF organization. In this study, keratins 18, 8, and 19 were identified to interact with the amino end domain of pinin in a two-hybrid screening. Further truncation analyses indicated that the 2B domain of keratin contains the sequence responsible for interacting with pinin. The amino end of pinin (residues 1–98) is sufficient to bind to keratin. Point mutation analyses revealed two essential residues within the pinin fragment 1–98, leucine 8 and leucine 19, for the interaction with keratin. Finally, in vitro protein overlay binding assays confirmed the direct interaction of the amino end domain of pinin with keratins, while pinin mutant L8P GST fusion protein failed to bind to keratin in the overlay assay. Coupled with our previous morphological observations and transfection studies, these data suggest that pinin may play a role in epithelial cell adhesion and the IF complex through a direct interaction with the keratin filaments.

Pinin was first identified to be a protein associated with the desmosome, which was recruited to the preformed desmosomes of the epidermis but absent at nascent desmosomes (1). Immunofluorescence and immuno-EM studies have shown pinin decorating keratin filaments near the cytoplasmic face of the desmosomal plaque in the vicinity of keratin filament convergence upon the desmosome. Our previous studies have revealed a correlation between the placement of pinin at the desmosome and an increase in the organization/stabilization of desmosome-IF complex (1, 2). Presumably, one of the functions of pinin is related to the desmosome-IF complex.

The expression level of pinin has been correlated with the overall epithelial phenotype. HEK-293 cells, when transfected with pinin full-length cDNA, exhibited a striking phenotype change from a fibroblast-like spindle shape to cells with extensive cell-cell contact growing in culture as islands (2). Intriguingly, EM analysis of these transfected cells revealed that the array of epithelial cell junctions was enhanced. In addition, carcinoma-derived cells, when transfected with pinin cDNA, exhibited inhibition of anchorage-independent growth in soft agar. Furthermore, pinin's gene locus and dysregulation of pinin expression in primary tumor tissues suggest that pinin may function as a tumor suppressor in certain types of cancer (3, 4).

Pinin has also been localized in the nucleus in various tissues as well as in cultured cell lines (5–7). Brandner et al. (6) has proposed an involvement of pinin in spliceosomal function. The dual location of pinin may be indicative of the involvement of pinin in multiple cellular activities, both at the desmosome and in the nucleus; however, it is not yet clear whether or not the function of pinin in cell-cell adhesion is coordinated with its function in the nucleus. As a step toward understanding the functions of pinin, we sought to identify proteins that interact with pinin. In this study, we focus on the ability of pinin to bind keratin.

Keratin filaments are anchored to the lateral plasma membrane at desmosomes. These intercellular junctions reinforce epithelial adhesion as well as integrate the IF network across the entire epithelium. Numerous structure-function studies of desmosomal proteins have revealed details pertaining to the molecular organization of desmosome-IF complex. The relationships among the desmosomal components have been extensively reviewed elsewhere (8–11). The constitutive components of the desmosome include desmosomal cadherins (desmogleins and desmocollins) and plaque proteins, plakoglobin, desmoplakin, and plakophilin. Among these proteins, desmoplakin (12, 13) and plakophilin (11, 14) have been shown to bind directly to keratins. In addition, other peripherally associated desmosome proteins such as plectin (15, 16), envoplakin/periplakin (17, 18), and pinin (1) are also thought to interact, directly or indirectly, with keratin. Significant questions pertaining to the molecular associations and specific roles of these accessory proteins of the desmosome remain.

To identify potential protein-protein interactions of pinin, a two-hybrid screening was performed with either the amino portion or the carboxyl portion of pinin as bait. In this study, we presented a detailed analysis on the binding of the amino end domain of pinin to one group of the identified proteins, the keratins. Keratin 18 (K18), keratin 8 (K8), and keratin 19 (K19) were shown to interact with the amino portion of pinin in the two-hybrid screen. Further truncation analyses defined the specific domain of keratin that mediates the interaction. In addition, the specific domain of pinin molecule sufficient for the interaction was characterized, and through site-directed mutagenesis, the essential residues within this particular domain were investigated. In vitro blot overlay assays were performed...
to confirm the interaction between the amino end domain of pinin and the keratins. Overall, our data strongly suggest that pinin is capable of binding directly to the intermediate filament proteins, specifically the keratins. These data provide important information on eventual understanding of mechanism by which pinin may affect the assembly/stabilization of epithelial cell adhesion.

MATERIALS AND METHODS

Yeast Strain and Media—The Saccharomyces cerevisiae strain PJ69–4A (25) trp1–901 leu2–3, 112 ade2–100 his3–200 leu2–3, 112 ura3–52 was used for all the two-hybrid assays. The yeast was grown on synthetic medium (SD) with appropriate amino acid-omissions for plasmid selection. Trypophan and leucine were selective markers for the co-transformed bait and prey plasmids. Histidine 3, adenine 2, and lacZ are reporter genes for interaction between GALA-BD and GALA-AD. In “His’’ medium, histidine was omitted as well as tryptophan and leucine. In addition, 1 M 3-aminotryptocephalin was added in all of the media to inhibit the autoactivation of the histidine 3 reporter gene.

Bait Construct and Two-hybrid Screening—The DNA fragment encoding for pinin residues 1–480 was obtained by PCR and cloned in-frame between the DNA binding domain (DBD) GALA-BD (residues 9–289) in the pAS2–1 (CLONTECH, Matchmaker II system). The GALA-BD/pincoin vector was co-transformed with a CLONTECH Matchmaker cDNA library into the yeast strain PJ69–4A using the yeast transformation method of Gietz et al. (20). The library consisted of human fetal kidney cDNA fused to the activation domain of GAL4 (GALAAD, prey) in the pGAD 10 vector (CLONTECH).

Approximately 10⁶ transformants were screened. They were then initially subjected to His selection; subsequently, the colonies of surviving yeast were replicated to Ade plates. Positive colonies from Ade selection were subjected to liquid culture ONPG β-galactosidase assays according to the manufacturer’s procedure (CLONTECH). The interaction between p53 and SV40 large T-antigen was used as a positive control in β-galactosidase assays according to the manufacturer’s procedure. The base-line level of β-galactosidase activity was determined from control yeast co-transformed with GALA-BD-pinoin (residues 1–480) and GALA-AD. Each reported value of β-galactosidase units represented an average enzyme activity determined from three independent colonies. The “prey” plasmids were recovered from triple positive (His’, Ade, and LacZ) clones and co-transformed with the GALA-BD in the pGAD10 vector (CLONTECH).

To examine the ability of truncations of pinin to interact with keratin, the GALA-BD vectors containing the individual pinin truncations or point mutation constructs were co-transformed with the pGAD10 vector containing keratin 18 into PJ69–4A yeast. To examine the ability of truncations of keratin 18 to interact with the amino end of pinin, the original bait was co-transformed with individual truncations of keratin 18 to interact with the amino end of pinin, the amino portion domains.

Expression of Pinin Fusion Protein in Escherichia coli and Generation of the Polyclonal Antibody against the Pinin GST-Fusion Protein—Pinin residues 1–165 were obtained by PCR with primers 5’-CCG AAT TCC CGC AGC TGA GAG AGT-3’ and 5’-CCG TCG AGG GCC TTT CAG TAG CAA CAG-3’. This PCR fragment was cloned in frame to vector pGEX-4T-3 (Amersham Pharmacia Biotech) at Xhol/EcoRI sites. The glutathione S-transferase (GST) fusion protein GST-cp-(1–165) was expressed in E. coli strain BL21 (Novagen) and purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Specifically, a mutant GST fusion protein of the residues 1–165, GST-cp-(1–165) LSP, with a substitution of leucine 8 by a proline, was generated with the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), expressed and purified as described above.

The pinin DNA encoding for 5′-end residues 1–165 was also cloned into pET 28 (+) (pET system; Novagen) and expressed as a T7-tagged and His6 fusion protein in E. coli strain BL21 (Novagen). The fusion protein was affinity-purified using the charged His-Bind metal chelation resin (Ni2+ beads) following the instructions of the manufacturer (Novagen, pET System Manual).

A rabbit polyclonal antibody (UF215) was generated using the GST-cp-(1–165) as antigen (Cocalico Biologicals, Inc.). The specific immune activity of UF 215 to pinin’s amino domain was verified by Western blot on pET System expressed His6 fusion protein described above (data not shown).

Purification of Keratin Filament Protein from Madin-Darby Canine Kidney Cells—Madin-Darby canine kidney cells were grown to confluence in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM gluta mine and 200 units/ml each of penicillin and streptomycin. Keratin proteins were then prepared from these cells according to a procedure described elsewhere (21, 22) with slight modifications. Cells were lysed in PBS (containing 1% Triton X-100, 0.6 M KCl, 1 mM MgCl2, 5 mM EDTA, 5 mM EGTA, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 1 mg/ml leupeptin, 1 mM mg/ml pepstatin A, 1 mg/ml aprotinin (Sigma)). The extract was treated with DNase (0.5 μg/ml) at 37 °C for 20 min and then centrifuged at 2000 × g at 4 °C for 10 min to pellet the IF-enriched cytoskeleton. The IF-enriched cytoskeletal preparation was first extracted with PBS containing 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, sequentially washed with PBS, and finally extracted with salt buffer (60 mM KCl, 1 mM EDTA, 1 mM cysteine, 10 mM ATP, 40 mM imidazole, pH 7.1), high salt buffer (0.6 M KCl, 1 mM EDTA, 2 mM ATP, 1 mM cysteine, 40 mM imidazole), and low salt buffer again. This KCl-extracted pellet was dissolved in 8 μl urea in 10 mM Tris-HCl buffer supplied with protease inhibitors and subjected to ultracentrifugation at 125,000 × g for 1 h at 4 °C. The supernatant was dialyzed into 10 mM Tris-HCl and frozen at −80 °C.

In Vitro Blot Overlay Binding Assays—In vitro overlay protein binding assays were performed as described elsewhere with slight modification (11). 2 μg of purified keratin, bovine serum albumin, pinin amino acid fragment 1–165, and mutant pinin 1–165 LSP were separated on a 10% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes. Membranes were blocked by incubation in reaction buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl2, pH 7.4) with the addition of 0.1% (v/v) Tween 20 and 5% (v/v) nonfat milk powder at 4 °C overnight. Blots were incubated 4 h at room temperature with the bacterially expressed pinin amino domain, either wild typeGST-cp-(1–165) or mutant GST-cp-(1–165) LSP (3 μg/ml in the reaction buffer with the addition of protease inhibitor mixture (Boehringer Mannheim) and 0.1% Tween 20, 1% bovine serum albumin, and 0.5% Triton X-100). After incubations, the blots were washed thoroughly with several fresh changes of the reaction buffer and subjected to routine Western blotting with anti-pinin antisera and ECL (Amersham Pharmacia Biotech). Specifically, UF215 diluted 1:1000 in TBS (10 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl2, 0.1% Tween 20, pH 7.4) was used as the primary antibody. Blots were incubated in 5% normal goat serum in TBS prior
to secondary antibody (goat anti-rabbit IgG, Amersham Pharmacia Biotech; 1:10,000) incubation. As a control for the protein overlays, GST was used instead of wild type pinin fusion protein and subsequently probed with anti-GST antibody (Amersham Pharmacia Biotech) via Western blot.

**RESULTS**

**K18, K8, and K19 Were Identified in a Yeast Two-hybrid Screening by the Amino Portion Fragment of Pinin**—In an effort to identify proteins that bind to the amino-terminal domain of pinin, a yeast two-hybrid screening on a human fetal kidney cDNA library (CLONTECH) using pinin (residues 1–480) as bait was performed. Of the approximately 10^6 transformants screened, 21 independent cDNA clones were isolated. The recovered prey plasmids were verified by co-transforming formants screened, 21 independent cDNA clones were isolated.

The data suggest that either some sequence outside the coil 2 domain may contribute to the interaction or that the longer constructs may present the binding domain of keratin in a more advantageous conformation for pinin binding.

**The 2B Domain of Keratin Contains the Binding Site for Pinin**—K18, K8, and K19 are three keratins expressed in the simple epithelial cells. These keratins share common structural properties. Each possesses an amino end nonhelical head domain, a central coiled-coil α-helical domain, and a nonhelical tail domain in various lengths (23, 24). Because pinin (residues 1–480) bound equally well to each of these keratin clones and the common domain shared by all of the clones was the rod domain, we surmised that the rod domain might contain the sufficient sequence for the interaction with pinin. To further map the binding site within keratin, truncation constructs coding either coil 1 or coil 2 of K18/K8/K19 were generated and examined for their ability to bind pinin in a two-hybrid assay (Fig. 1). While constructs containing the coil 1 domain of K18 (residues 69–240), K19 (residues 81–235), and K8 (residues 91–235) exhibited no significant binding to pinin (residues 1–480), the coil 2-containing constructs of K18 (residues 234–391), K19 (residues 244–390), and K8 (residues 260–381) all exhibited interaction with pinin. It was, however, noticed that the coil 2-pinin interactions were approximately 10-fold weaker than the interaction of the intact keratin rod domain as indicated by the β-galactosidase assay. While reporter gene activity, such as β-galactosidase, does not correspond linearly with the strength of interaction, these assays can be useful in estimating relative strength of interactions between similar molecules or domains. The data suggest that either some sequence outside the coil 2 domain may contribute to the interaction or that the longer constructs may present the binding domain of keratin in a more advantageous conformation for pinin binding.

The carboxyl terminus of the 2B domain within coil 2 contains a highly conserved consensus motif, suggested to be significant for assembly/stabilization of the intermediate filaments in cells (25–28). K18 (residues 69–276), which excluded the entire 2B domain, failed to interact with pinin (residues 1–480). However, K18 (residues 69–372), which contained the majority of the rod domain but not the consensus motif, retained the ability to bind to pinin (residues 1–480) (Fig. 2). Considering this, together with the results shown in Fig. 1, we conclude that the 2B domain of keratin contained the binding site for pinin.

**Pinin Residues 1–98 Are Sufficient for Interacting with Kera-
The amino end of pinin (residues 1–480) contains a short domain with heptad repeats, a few glycine loops (29), and a rather extensive glutamate-rich a-helix domain (2). To more precisely map the domain of pinin that is sufficient for the interaction with keratin, five pinin truncation constructs were generated for two-hybrid analyses (Fig. 3). Constructs lacking the amino terminus of pinin (residues 85–480, 250–480, and 85–252) exhibited no significant interaction with keratin, while constructs (residues 1–252 and residues 1–98) containing amino end heptad repeats and glycine loops exhibited binding to keratin.

Leucine 8 and Leucine 19 within Pinin Are Essential for Binding to Keratin—To further define the specific region within the amino end of pinin that is essential for binding to keratin, site-directed mutagenesis was employed. Leucine residues at positions 8, 19, and 29, which were predicted to locate at either the “a” or “d” position of the heptad repeats within pinin (30–32), were substituted with proline (N_8L_8P, N_9L_19P, and N_9L_29P). Interestingly, both N_8L_8P and N_9L_19P resulted in no growth at all on 2Ade medium (Fig. 4A) and a base line level of β-galactosidase activity (Fig. 4B), indicating the interaction between pinin and K18 was abolished with a single mutation. On the contrary, N_9L_29P retained the ability to grow on –Ade medium, but the β-galactosidase activity was somewhat reduced. One glycine within the predicted first glycine loop of pinin was replaced by glutamate (N_9G_53Q). This substitution, similar to N_9L_29P, did not affect the growth of transformed yeast under selection conditions, but it resulted in a somewhat weaker interaction, as indicated by reduction in β-galactosidase activity.

**Fig. 1.** Two-hybrid analyses demonstrated that the coil 2 within the rod domain of K18/K8/K19 contained sufficient sequence to bind to the amino-terminal domain of pinin hp-(1–480). Human pinin (residues 1–480) fused to GAL4-BD and one of the keratin constructs fused to GAL4-AD were cotransformed into the yeast host strain PJ69–4A. Transformants were selected on –HIS, –Ade, and β-galactosidase selection assays. A, yeast containing pinin N_bait hp-(1–480) and one of the coil 2 constructs, K18-(234–391), K8-(260–388), and K19-(244–390), exhibited growth on –Ade selective medium SD/–Trp, –Leu, –Ade, while yeast containing hp-(1–480) and one of the coil 1 constructs, K18-(69–240), K8-(91–235), or K19-(81–229), exhibited no growth. B, β-galactosidase activity (β-gal units) obtained from quantitative β-galactosidase assay of each transformant confirmed the results from –Ade selection assay, that the coil 2 domain and hp-(1–480) interacted with each other to activate the lacZ gene, while no interaction occurred between the coil 1 domain of K18/K8/K19 and hp-(1–480).

**Fig. 2.** Two-hybrid analyses defined the 2B domain of keratin 18 interacting with pinin amino portion 1–480. Either the 2B consensus motif deletion construct K18(69–372) or the 2B domain deletion construct K18(69–276) was cotransformed into yeast PJ69–4A with hp-(1–480). The cotransformants were selected on –HIS, –Ade medium and subjected to β-galactosidase (β-gal) assay. A, yeast containing K18 (69–372), as well as yeast containing full-length K18 exhibited growth on SD/–Trp, –Leu, –Ade medium, while the yeast containing K18 (69–276) exhibited no growth. B, β-galactosidase assays indicated that K18-(69–372) is able to bind to hp-(1–480), while K18-(69–276) exhibited no binding to hp-(1–480).

**Fig. 3.** Two-hybrid analyses mapped the site in pinin for interacting with keratin 18. Human pinin constructs were cotransformed with K18 into yeast PJ69–4A. As indicated by growth on selective medium SD/–Trp, –Leu, –Ade medium and subjected to β-galactosidase (β-gal) activity (B), pinin fragment hp-(1–98) contained the predicted heptad repeat and glycine loop domains, is sufficient for the interaction of pinin with keratin 18 and N_9L_29P. Interestingly, both N_9L_8P and N_9L_19P resulted in no growth at all on –Ade medium (Fig. 4A) and a base line level of β-galactosidase activity (Fig. 4B), indicating the interaction between pinin and K18 was abolished with a single mutation. On the contrary, N_9L_29P retained the ability to grow on –Ade medium, but the β-galactosidase activity was somewhat reduced. One glycine within the predicted first glycine loop of pinin was replaced by glutamate (N_9G_53Q). This substitution, similar to N_9L_29P, did not affect the growth of transformed yeast under selection conditions, but it resulted in a somewhat weaker interaction, as indicated by reduction in β-galactosidase activity.
β-galactosidase activity. Charged residues have been speculated to stabilize coiled-coil conformations. However, changes of arginine 6 and lysine 28 to aspartate and glutamate, respectively (N^L8P, N^K28E), resulted in only a slight dampening in the β-galactosidase activity (Fig. 4). In summary, leucine 8 and 19 were shown to be essential for pinin-keratin interaction, whereas leucine 29, glycine 53, arginine 6, and lysine 28 were not essential but may somehow be involved in the optimal pinin-keratin interaction. Whether or not multiple (additive) substitutions of the residues would result in a more obvious effect on the pinin-keratin interaction is currently under investigation.

In Vitro Overlay Binding Assays Verified the Direct Interaction between Pinin Amino End Domain and Keratins—Keratin, purified from Madin-Darby canine kidney cells and bacterially expressed pinin fragments, both wild type GST-cp-(1–165) and mutant GST-cp-(1–165) L8P, were utilized in the blot overlay binding analyses. Blots containing keratin preparations were overlaid with either wild type GST-cp-(1–165) L8P and hp-(1–480), while N^L29P, N^G53Q, N^R6D, and N^K28E remained to interact with hp-(1–480).

In this study, we present data demonstrating the direct interaction of the amino end domain of pinin with the 2B domain of keratin from simple epithelial cells. These data are not only consistent with our previous morphological observations but provide biochemical support of pinin-IF association.

There are four distinct coiled-coil stretches, 1A, 1B, 2A, and 2B in the central rod domain of a keratin molecule. Our data indicate that pinin binds to the sequence within the 2B domain of keratin. Coil 1 of keratin exhibited no binding to pinin, strongly supporting the conclusion that the interaction between the 2B domain of keratin and pinin amino-terminal domain is indeed specific and not due to nonspecific interaction with coiled-coil-containing proteins. Direct binding to the rod 2B domain of keratin 18 has been reported for BPAG 2, a hemidesmosome-associated protein (33). While desmoplakin has been shown to bind to the head domain of epidermal keratins, such as keratin 1/keratin 10 and keratin 5/keratin 14 (11), it has also been shown to be capable of binding to the rod domain of simple epithelial keratin K8/K18 heterodimer (13).

The truncation analyses suggested the amino end domain of pinin (residues 1–98) contained the sequence responsible for binding to keratin. Although short coiled-coils composed of four to five heptad repeats have been reported (31), it is not clear whether the four and a half heptad repeats at the amino end of pinin are actually sufficient to form a coiled-coil structure in vitro. The amino end domain of pinin does not contain a “trigger sequence” (34), so
it may not participate in the formation of a coiled-coil. However, data derived from point mutation analyses of the aminoterminal domain of pinin suggest the sequence within the heptad repeats is indeed essential for the interaction with keratin. N’ L8P and N’ L19P completely abolished the binding of pinin to K18, whereas N’ L29P did not, suggesting that the heptad repeats located nearer the amino end of pinin may play a more significant role in pinin-keratin interaction.

We have suggested that pinin may function as a tumor suppressor based on chromosomal location of pinin and tumor biological analyses (4). It has been shown that the expression of pinin was absent or greatly reduced in certain carcinomas, including renal cell carcinoma and transitional cell carcinoma. On the other hand, pinin expression was up-regulated in a subset of melanoma tissues (3) and a subset of renal cell carcinoma (4). Decreased expression of pinin was correlated with loss of epithelial cell-cell adhesion, while increasing pinin expression by transfection of pinin cDNA was shown to enhance cell-cell adhesion (4, 35). Interestingly, K18 and K8 have long been considered as cytological markers for carcinomas due to their persistent expression in tumor cells derived from simple epithelia and their aberrant expression in malignant progression of nonepithelial cells (36–39). In addition, several studies suggested that K18/K8 filaments have a role in the tumorigenicity. For example, in K8-deficient mice, adult animals developed pronounced colorectal hyperplasia (40), and the expression of K8 and K18 in human melanoma cell lines resulted in increased invasive and metastatic properties of the cells (37, 41). It is tempting to speculate that the tumor suppressor function of pinin is related to the interaction of pinin with keratin.

This study did not address the important issue regarding the relationship between the desmosome and pinin. Our initial two-hybrid screens identified other, as of yet uncharacterized, proteins interacting with pinin N’ bait 1–480.2 One of these clones coded for a protein containing motifs highly homologous to periplakin (18), a desmosome-IF-associated protein suggested to be analogous to periplakin (18), a desmosome-IF-associated protein including renal cell carcinoma and transitional cell carcinoma. We have suggested that periplakin-like protein is currently being addressed.

In summary, we have demonstrated that pinin can bind to keratin 18, keratin 8, and keratin 19. The 2B domain of keratin contains the sequence mediating the interaction with pinin, and the amino end (residues 1–98) of pinin was sufficient to bind keratin. Identification of specific binding sites within pinin for keratin and for other proteins will be an integral step for future studies. We believe that investigation of the function(s) of pinin in cell adhesion and IF organization will greatly contribute to our current knowledge of epithelial cell-cell adhesion.

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