Journal of Cellular Biochemistry 96:390–403 (2005)

A Role for Plakophilin-1 in the Initiation of Desmosome Assembly

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Abstract Plakophilins (pkp-1, -2, and -3) comprise a family of armadillo-repeat containing proteins that are found in the desmosomal plaque and in the nucleus. Plakophilin-1 is most highly expressed in the suprabasal layers of the epidermis and loss of plakophilin-1 expression results in skin fragility-ectodermal dysplasia syndrome, which is characterized by a reduction in the number and size of desmosomes in the epithelia of affected individuals. To investigate the role of plakophilin-1 during desmosome formation, we fused plakophilin-1 to the hormone-binding domain of the estrogen receptor to create a fusion protein (plakophilin-1/ER) that can be activated in cell culture by the addition of 4-hydroxytamoxifen. When plakophilin-1/ER was expressed in A431 cells it was incorporated into endogenous desmosomes and did not disrupt desmosome formation. A derivative of A431 cells (A431D) do not form desmosomes, even though they express all the components believed to be necessary for desmosome assembly. Expression and activation of plakophilin-1/ER in A431D cells resulted in punctate desmoplakin staining on the cell surface. Co-expression of a classical cadherin (N-cadherin) and plakophilin-1/ER in A431D cells resulted in punctate desmoplakin staining at cell–cell borders. These data suggest that plakophilin-1 can induce assembly of desmosomal components in A431D cells in the absence of a classical cadherin; however a classical cadherin (N-cadherin) is required to direct assembly of desmosomes between adjacent cells. The activatable plakophilin-1/ER system provides a unique culture system to study the assembly of the desmosomal plaque in culture. J. Cell. Biochem. 96: 390–403, 2005.

Key words: plakophilin-1; desmosome; cell–cell adhesion

Desmosomes are prominent cell–cell adhesive structures found in a variety of epithelial tissues and are thought to allow cells to withstand mechanical stress. In addition to their adhesive function, desmosomes may also provide spatial cues to cells in a variety of epithelial tissues [Runswick et al., 2001; Garrod et al., 2002]. The transmembrane components of the desmosome are the desmosomal cadherins, desmoglein, and desmocollin. The cytoplasmic domain of the desmosomal cadherins are linked to the intermediate filament cytoskeleton through interactions with a group of proteins that comprise the desmosomal plaque. Plakoglobin and desmoplakin are constitutive desmosomal plaque proteins while the plakophilins cell type specific desmosomal plaque proteins.

Plakophilins (plakophilin-1, -2, and -3) are armadillo repeat containing proteins that were originally identified as desmosomal plaque proteins and subsequently shown to also have nuclear functions [Hatzfeld et al., 1994; Mertens et al., 1996; Schmidt et al., 1997]. The plakophilins share a high degree of amino acid similarity with the adherens junction protein p120, and consist of a unique amino terminal head domain and 9 armadillo repeats [Choi and Weis, 2005]. Plakophilin-1 is abundant in stratified epithelia and is most prominently expressed in the suprabasal layers of the skin [Moll et al., 1997].

The amino terminal head domain of plakophilin-1 interacts directly with keratin intermediate filaments, desmoglein 1 [Hatzfeld et al., 2000] and desmoplakin [Kowalczyk et al., 1999]

Grant sponsor: NIH (to JKW); Grant number: P20 RR018759.

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Received 2 March 2005; Accepted 31 March 2005

DOI 10.1002/jcb.20514

and is thought to stabilize the desmosomal plaque by promoting the lateral assembly of the desmosomal cadherins in the plasma membrane [Bornslaeger et al., 2001] and recruiting desmoplakin to sites of cell–cell contact [Kowalczyk et al., 1999; Hatzfeld et al., 2000]. An essential role for plakophilin-1 in desmosomal adhesion is confirmed by the finding that patients with truncating mutations in plakophilin-1 suffer from ectodermal dysplasia and skin fragility syndrome [McGrath et al., 1997], which is characterized by cutaneous fragility, painful palmoplantar fissuring, nail dystrophy, and perioral lesions [Hamada et al., 2002]. Ultrastructural examination of the epithelia from these patients showed decreased numbers of desmosomes and reduced desmosome size, suggesting that plakophilin-1 is important for assembly of mature desmosomal plaque structures and maintenance of strong cell–cell adhesion. In addition, keratinocytes from these patients exhibit increased motility, presumably due to decreased desmosome assembly [South et al., 2003].

In the present study, we investigated the role of plakophilin-1 in desmosome assembly. We constructed an activatable form of plakophilin-1 by fusing plakophilin-1 to the ligand binding domain of the estrogen receptor (plakophilin-1/ER). The fusion protein was rapidly activated by addition of 4-hydroxytamoxifen to the culture media when we expressed it in squamous epithelial cells, which allowed us to assess the function of plakophilin-1 in desmosome assembly. Although plakophilin-1 is not an obligatory component of the desmosomal plaque, we demonstrate here that plakophilin-1 can initiate the assembly of desmosomes in cells that were previously unable to assemble these structures.

MATERIALS AND METHODS

Cell Culture

A431 cells were obtained from ATCC (Manasass, VA) and HaCat cells were a gift of Dr. Pamela Jensen (University of Pennsylvania, Philadelphia, PA). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). A431D and A431DN cells have been described [Lewis et al., 1997; Kim et al., 2000].

Generation of cDNA Constructs

A plakophilin 1a cDNA was generated by RT-PCR from human keratinocyte total RNA using the upstream primer 5′ TAGAATTCTCTCC-TAGGCCCCGGCC-3′ and the downstream primer 5′-CTAAGCTTTAACTTGCTTGG-3′. The resulting PCR product was subcloned into pUC19 and sequenced to verify that the cDNA encodes wild type plakophilin 1a (genbank accession #Z73678). To produce retrovirus encoding plakophilin-1 the plakophilin-1 cDNA was digested with EcoRI and HindIII, filled in using Klenow enzyme, and ligated to LZRS digested with EcoRI and AfeI. To produce plakophilin-1/ER, we used PCR to remove the stop codon and introduce an XhoI site (5′-CT-TCACCTCCCGATTCCTCGAGGGGCGGCCG-C-3′ and 5′-TCCAGCAGCATGCTCAACAAC-3′). The mutant estrogen receptor ligand binding domain (ER^{T2}) [Feil et al., 1997] was a kind gift from Dr. Pierre Chambon (Strasbourg, France). ER^{T2} was ligated to plakophilin 1 in pUC19 as an XhoI/NotI fragment to generate plakophilin-1/ER. The resulting cDNA was subcloned into LZRS-Neo [Ireton et al., 2002] as an EcoRI fragment and used to produce retrovirus encoding plakophilin-1/ER.

Retrovirus Production and Infection

Retrovirus production and infection was performed essentially as described by Ireton et al. [2002]. Briefly, phoenix cells (5×10^5 per 100 mm dish) were transfected with constructs prepared in the LZRS vector using calcium phosphate (Stratagene), 48 h following transfection cells were switched to media containing 2 μg/ml puromycin (Sigma) to select for virus producing cells. Populations of puromycin resistant cells were grown in DMEM supplemented with 10% FBS lacking puromycin and grown at 32°C for 24 h for virus production. Viral conditioned media was collected and passed through a 0.45 μm syringe filter and polybrene (Sigma) was added to 4 μg/ml. For infection of target cells, 2×10^5 cells were plated in one well of a six well dish 18 h prior to infection. Fresh viral conditioned media was incubated with targets cells for 6 h at 32°C. Following infection, the media was replaced with fresh media and cells were returned to 37°C. Two days after infection selective media containing 1 mg/ml G418

was added and a population of cells expressing the desired protein product was isolated.

Detergent Extraction of Cells and Immunoblot Analysis

For total cell lysates, cells grown in T25 cm^2 flasks were grown to confluency, rinsed three times with phosphate buffered saline containing 2 mM sodium orthovanadate and extracted in 1 ml Empigen BB extraction buffer (10 mM Tris HCl pH 7.0, 0.1% Empigen BB (Calbiochem), 5 mM EDTA, 2 mM EGTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride). Cells were placed on ice, scraped, and triturated vigorously for 10 min. Insoluble material was pelleted by centrifugation at 14,000*g* for 15 min at 4°C, and the supernatant was used immediately or stored at −70°C. Protein concentration was determined using DC protein assay (BioRad, Hercules, CA). To determine NP-40 solubility, cells were extracted in NP-40 buffer (10 mM Tris HCl, pH 7.0 0.5% NP-40, 2 mM EDTA and 2 mM phenylmethylsulfonyl fluoride), insoluble material was pelleted by centrifugation at 14,000*g* for 15 min at 4°C. The NP-40 soluble fraction was removed and the insoluble pellet was resuspended in a volume of Laemmli sample buffer equal to the volume of NP-40 extraction buffer used. Cell extracts were prepared in Laemmli sample buffer [Laemmli, 1970], boiled for 5 min, and the proteins were resolved by SDS–PAGE. Proteins were electophoretically transferred overnight to nitrocellulose membranes and blocked in 5% non-fat dry milk in TBST (10 mM Tris HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 45 min. Blocking solution was removed by washing for 15 min followed by 2×5 min in TBST. For immunoblots, hybridoma conditioned media was used at 1:100 dilution in TBST for 1 h. Membranes were washed 15 min followed by 2×5 min in TBST. Membranes were incubated with horseradish peroxidase conjugated anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA) at 1:10,000 for 1 h. Secondary antibody was removed by washing 15 min followed by 4×5 min in TBST. Immunoreactive bands were detected using Super Signal Pico substrate (Pierce Chemical Co., Rockford, IL).

Antibodies

A plakophilin-1 monoclonal antibody (14B11) directed against the amino terminal head domain was produced as previously described [Johnson et al., 1993; Wahl, 2002] using a maltose binding protein fusion containing amino acids 1–234 of human plakophilin-1. Anti desmoglein 2 (6D8), anti-desmocollin 2 (7G6), anti-plakoglobin (11E4) have been described [Wahl et al., 1996; Wahl, 2002]. Rabbit anti-desmoplakin (NW6) was a kind gift of Dr. Kathleen Green (Northwestern University, Chicago, IL). Mouse anti-desmoplakin multiepitope cocktail, anti-plakophilin-2 and anti-plakophilin-3 antibodies were purchased from Research Diagnostics Inc. (Flanders, NJ).

Immunofluorescence Microscopy

To visualize plakophilins, cells grown on glass cover slips were washed briefly in HEPES buffered Hanks' balanced salt solution and fixed in methanol at −20°C for 5 min followed by acetone for 30 s. Coverslips were briefly air dried and washed three times in phosphate buffered saline containing 0.0001% Triton X-100 (PBST) before blocking in PBST and 10% heat inactivated goat serum. Alternatively, cells were fixed in 1% paraformaldehyde and permeabilized using 0.2% Triton X-100 in phosphate buffered saline and 10% goat serum. Cells were incubated in primary antibody for 1 h and primary antibody was removed by washing once in blocking solution and three times in PBST. FITC conjugated secondary antibodies were diluted (1:100) in blocking buffer and added to cells for 1 h, excess secondary antibody was removed by washing once in blocking solution and three times in PBST. Coverslips were briefly washed in distilled water and mounted using vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA). Images were collected on a Zeiss Axiovert 200M equipped with an ORCA-ER (Hamamatsu) digital camera. Images were collected and processed using OpenLab software from Improvision Inc. (Boston, MA).

Electron Microscopy

Cells were plated on Thermanox coverslips (Nalgene Nunc International, Rochester, NY) in six well dishes 4 days prior to fixation. Confluent cultures were fixed overnight using 2.5% glutaraldehyde in 1× phosphate buffered

saline and processed by The University of Nebraska Electron Microscopy Facility as previously described [Lewis et al., 1997].

RESULTS

In order to study the function of plakophilin-1 we constructed a plakophilin-1/estrogen receptor ligand binding domain (ER) fusion protein and expressed this protein in squamous epithelial cells (Fig. 1A). Fusion proteins containing the estrogen receptor hormone binding domain have been used extensively to study the function of various proteins by generating a fusion protein that is sensitive to ligand binding [Picard, 2000; Weng et al., 2002]. The estrogen receptor ligand binding domain used in this study contains three amino acid substitutions that render it insensitive to endogenous estrogens and phenol red in the culture media while conferring sensitivity to 4-hydroxytamoxifen (4OHT) [Feil et al., 1997]. To begin characterization of our plakophilin-1/ER construct, we

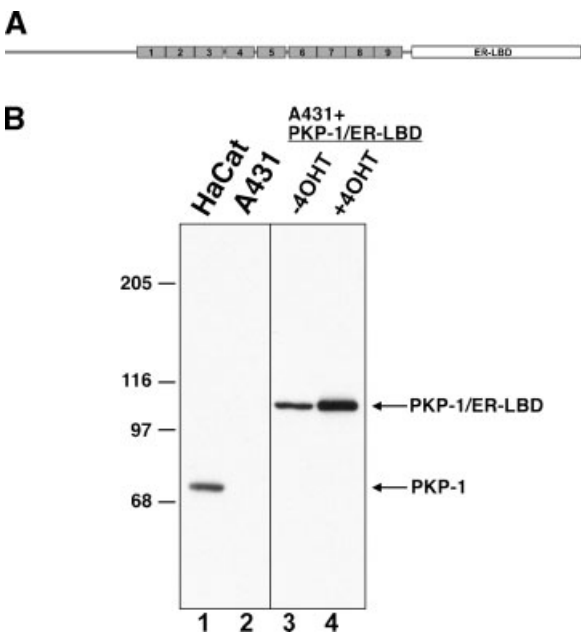

Fig. 1. Plakohilin-1/ER expression in A431 cells. **A**: A schematic diagram of the plakohilin-1/ER fusion protein used in this study. The estrogen receptor ligand binding domain has been fused to the carboxyl-terminus of human plakophilin-1a. **B**: Empigen BB cell lysates were prepared from HaCat cells (**lane 1**) A431 cells (**lane 2**), untreated A431 cells expressing plakophilin-1/ER (**lane 3**) and A431 expressing plakophilin-1/ER treated with 100 nM 4-hydroxytamoxifen for 16 h (**lane 4**). Equal protein from each cell lysate was resolved by SDS–PAGE, transferred to nitrocellulose, and immunoblotted using the anti plakophilin-1 monoclonal antibody 14B11. Note the lack of endogenous plakophilin-1 in A431 cells.

expressed the fusion protein in the squamous carcinoma cell-line A431. A431 cells express the desmosomal cadherins, desmoglein 2, desmoglein 3, desmocollin 2, and desmocollin 3 and the constitutive desmosomal plaque proteins desmoplakin and plakoglobin. These cells have previously been shown to form numerous desmosomes in culture [Wahl et al., 1996, 2000; Palka and Green, 1997]. In addition, our A431 cells express plakophilin-2 and plakophilin-3 but no detectable levels of plakophilin-1. Plakophilin-1/ER was introduced into A431 cells by retroviral infection [Ireton et al., 2002] and the expression of the fusion protein was verified by immunofluorescence and immunoblotting. For immunoblot analysis, whole cell lysates from A431 cells expressing plakophilin-1/ER, in the presence or the absence of 4-hydroxytamoxifen, parental A431cells, and HaCat cells were prepared in extraction buffer containing 0.1% Empigen BB. Under these conditions desmosomal proteins are completely solubilized [Wahl, 2002]. Cell lysate containing equal protein was resolved by SDS–PAGE, transferred to nitrocellulose, and immunoblotted using a monoclonal anti plakophilin-1 antibody. The monoclonal antibody recognized a single band in the HaCat extract that migrated at approximately 75 kDa, the predicted molecular weight of plakophilin-1 (Fig. 1B, lane 1). No signal was detected in A431 cells (Fig. 1B, lane 2) even after prolonged exposure (data not shown). Immunoblot analysis of the cell lysate prepared from A431 cells expressing plakophilin-1/ER revealed a single band migrating at 105 kDa, the expected molecular weight of plakophilin-1/ER (Fig. 1B, lanes 3 and 4). The levels of expression of the plakophilin-1/ER fusion protein were similar to the expression levels of plakophilin-1 found in HaCat cells. When A431 cells expressing plakophilin-1/ER were treated with 4-hydroxytamoxifen, the plakophilin-1 fusion protein level increased, presumably due to increased stability of the fusion protein, as documented for other ER fusion proteins [Weng et al., 2002].

Plakophilin-1/ER is Localized to the Desmosome and Nucleus Following 4-Hydroxytamoxifen Addition

Plakophilin-1 is found in the desmosome and in the nucleus in many squamous epithelial cells [Hatzfeld et al., 1994; Heid et al., 1994; Schmidt et al., 1997]. However, our A431 cells

do not express plakophilin-1 (Fig. 1B) and thus provide an excellent model in which to characterize the plakophilin-1/ER construct. It was important to first show that wild-type plakophilin-1 was properly localized when exogenously expressed in A431 cells. Thus, immunofluorescence was used to compare the subcellular localization of exogenously expressed plakophilin-1 in A431 cells (Fig. 2B) to that of endogenously expressed plakophilin-1 in HaCat cells (Fig. 2A). In both cell lines plakophilin-1 is localized to the nucleus and cell–cell borders. As expected, parental A431 cells showed no staining with anti plakophilin-1 antibody (Fig. 2C). Plakophilin-1/ER localization in A431 cells in the absence of 4-hydroxytamoxifen revealed a diffuse cytoplasmic staining (Fig. 2D). As early as 10 min following the addition of 4-hydroxytamoxifen, plakophilin-1/ER re-distributed to cell–cell borders and to the nucleus (Fig. 2E). Following 30 min of 4-hydroxytamoxifen treatment, a significant portion of the plakophilin-1/ER localized to the cell borders in a punctate distribution, indicative of desmosomal localization. In addition, plakophilin-1 was localized to the nucleus but excluded from nucleoli (Fig. 2F). These data suggest that plakophilin-1/ER is capable of incorporating into endogenous desmosomes in A431 cells and that the subcellular localization of plakophilin-1/ER following 4-hydroxytamoxifen is similar to that reported previously for wild-type plakophilin-1 [Schmidt et al., 1997; Klymkowsky, 1999; Kowalczyk et al., 1999; Hatzfeld et al., 2000].

The morphology of the cells expressing plakophilin-1/ER was unchanged following the addition of 4-hydroxytamoxifen suggesting that insertion of the fusion protein did not disrupt endogenous desmosomal structures. To confirm this we examined the subcellular distribution of the endogenous desmosomal components in cells expressing plakophilin-1/ER following 4-hydroxytamoxifen treatment. The distribution of desmoplakin, desmoglein 2, desmoglein 3, desmocollin, plakophilin 2, and plakophilin 3 was unaffected following 4-hydroxytamoxifen addition (Fig. 3). The adherens junction components, E-cadherin and β-catenin, also appeared to be unaffected by plakophilin-1/ER incorporation into the desmosome (Fig. 4).

Plakophilin-1/ER Expression in A431D and A431DN Cells

Our laboratory has made use of A431D cells, a derivative of A431 cells, to study desmosome assembly [Lewis et al., 1997]. A431D cells have lost expression of the classical cadherins, E- and P-cadherin, and do not exhibit calcium dependent cell–cell adhesion. These cells continue to express the desmosomal cadherins (dsg2, dsg3, dsc2, and dsc3) as well as desmoplakin and plakoglobin [Lewis et al., 1997] but do not form desmosomes. Previously we showed that simply reintroducing a classical cadherin was

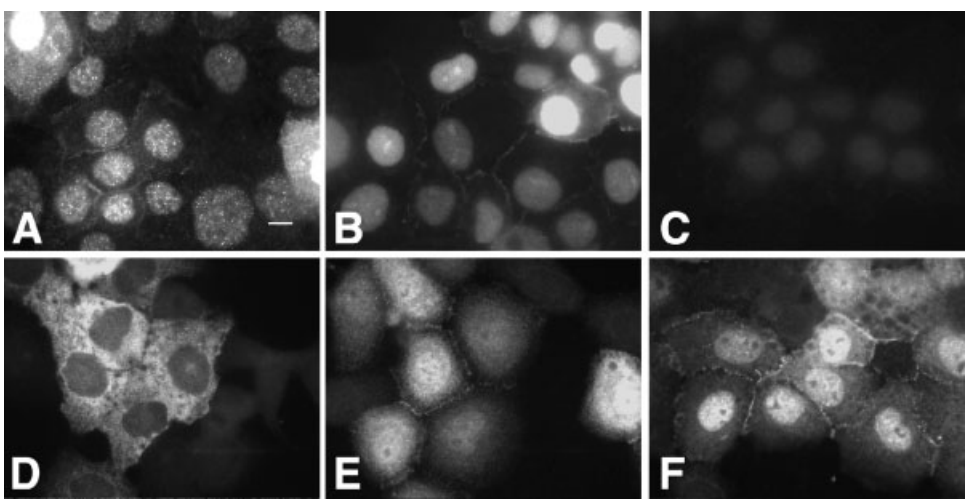

Fig. 2. Subcellular localization of plakophilin-1/ER in A431 cells. Cells were grown on glass coverslips and processed for immunolocalization of plakophilin-1 using anti-plakphilin-1 antibody (14B11). HaCat cells (**panel A**) endogenously express plakophilin-1. A431 cells were retrovirally infected with plakophilin-1 (**panel B**). Uninfected A431 cells do not express plakophilin-1 (**panel C**). A431 cells retrovirally infected with plakophilin-1/ER in the absence of 4-hydroxytamoxifen show diffuse cytosolic staining (**panel D**). A431 cells retrovirally infected with plakophilin-1/ER in the presence of 4-hydroxytamoxifen for 10 min (**panel E**) or 30 min (**panel F**) show nuclear and membrane staining.

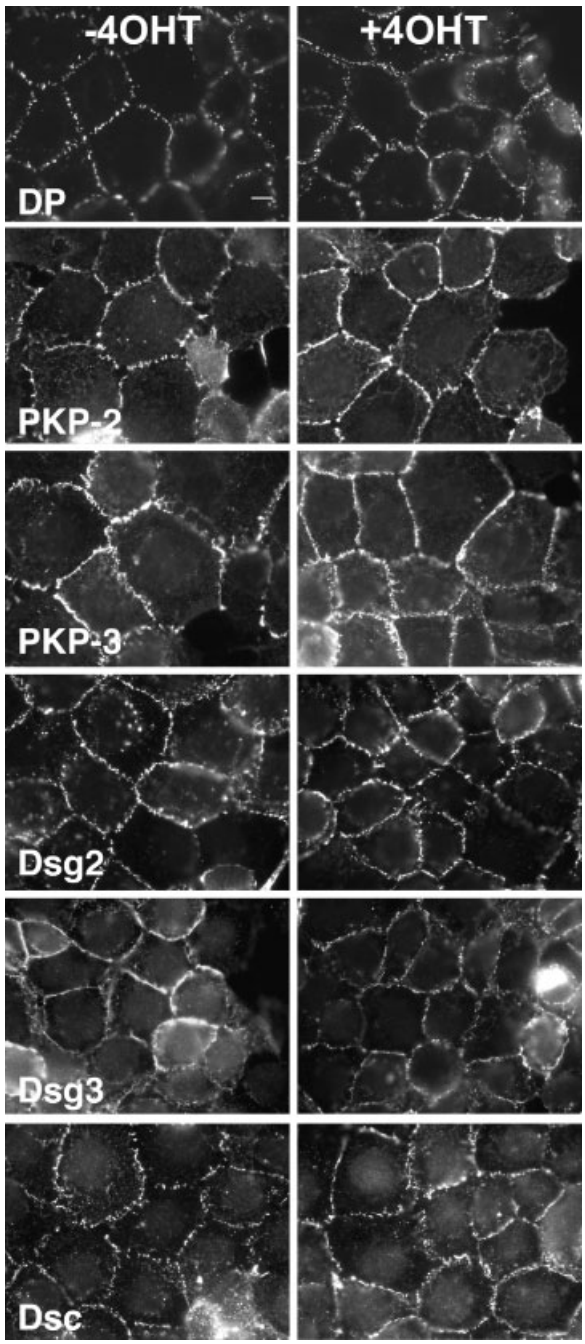

Fig. 3. Incorporation of plakophilin-1/ER into desmosomes does not alter the localization of desmosomal components. A431 cells expressing plakophilin-1/ER were processed for immunofluorescence with or without 100 nM 4-hydroxytamoxifen for 16 h. Cells were immunostained using mouse anti desmoplakin (**panels A** and **B**), anti plakophilin-2 (**panels C** and **D**), anti plakophilin-3 (**panels E** and **F**), anti desmoglein 2 (**panels G** and **H**), anti desmoglein 3 (**panels I** and **J**), or anti desmocollin (**panels K** and **L**).

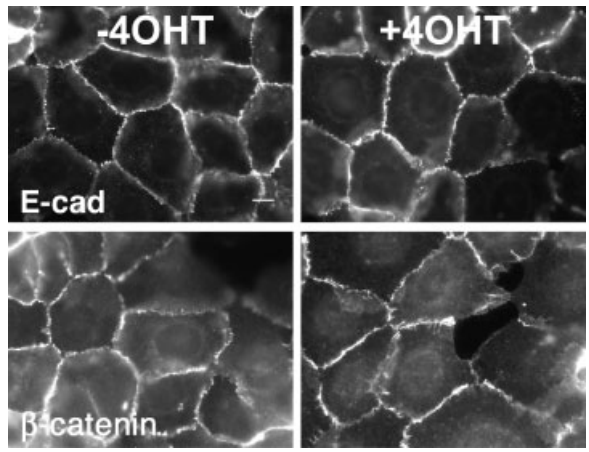

Fig. 4. Localization of adherens junction proteins are unaffected by plakophilin-1/ER activation. A431 cells expressing plakophilin-1/ER were processed for immunofluorescence with or without 100 nM 4-hydroxytamoxifen for 16 h. Cells were immunostained with anti E-cadherin (**panels A** and **B**) or anti β-catenin (**panels C** and **D**).

not sufficient to allow A431D cells to form desmosomes. However, when we increased plakoglobin expression, in addition to introducing a classical cadherin, A431D cells were able to form desmosomes.

In this study, we sought to investigate the role of plakophilin-1 in desmosome formation making use of A431D cells and A431D cells expressing the classical cadherin, N-cadherin (A431DN cells; [Kim et al., 2000]). Briefly, A431DN cells were generated by transfection using a cDNA encoding human N-cadherin and clones stably expressing N-cadherin were chosen. N-cadherin was chosen for the present study because it remains stably expressed in A431D cells after extended periods in culture (unpublished data). A particular clone of A431DN cells was chosen based on high level expression of desmoplakin, as judged by immunofluorescence (data not shown). Immunoblot analysis of whole cell lysates was performed to compare the expression of various desmosomal proteins in A431 cells, A431D cells, A431DN cells, and A431DN + plakophilin-1/ER (Fig. 5). Upon expression of N-cadherin in A431D cells the relative levels of plakoglobin, plakophilin-2, and desmocollin decreased slightly, and the level of plakophilin-3 increased slightly. The level of desmoglein 2 remained unchanged (Fig. 5 compare lanes 2 and 3). The differences in expression levels are most likely due to clonal variation following transfection of A431D cells with N-cadherin.

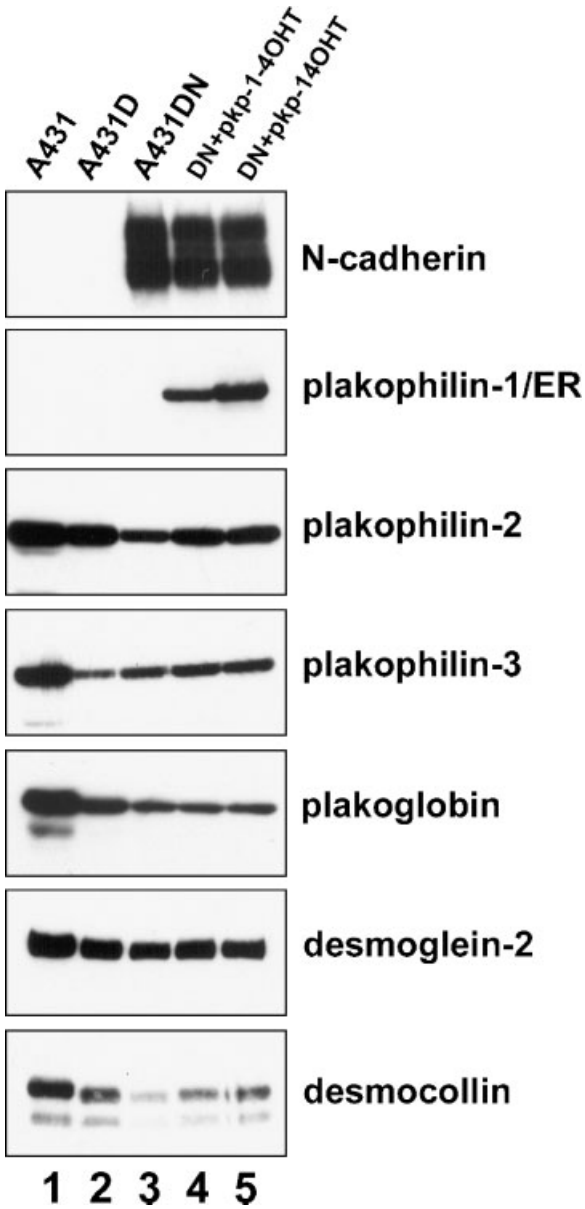

Fig. 5. Immunoblot analysis of cell junction components. Cell lysates were prepared from A431 cells (**lane 1**), A431D cells (**lane 2**), A431D cells expressing N-cadherin (A431DN) (**lane 3**), and A431DN cells expressing plakophilin-1/ER in the absence (**lane 4**) or the presence (**lane 5**) of 100 nM 4-hydroxytamoxifen for 16 h. Fifty of protein from each lysate was resolved by SDS–PAGE, transferred to nitrocellulose, and the membranes were blotted using antibodies specific for *N*-cadherin, plakophilin-1, plakophilin-2, plakophilin-3, plakoglobin, desmoglein 2, or desmocollin.

We next expressed plakophilin-1/ER in A431DN cells by retroviral infection and examined the expression levels of the desmosomal components. Plakophilin-2, plakoglobin, desmoglein 2, and desmocollin levels were comparable to A431D cells. Plakophilin-3 levels increased slightly (Fig. 5 compare lanes 2 and 4). Following the addition of 4-hydroxytamoxifen, plakophilin-1/ER protein levels increased to a similar extent as that seen in A431 cells. The expression level of the other desmosomal components remained unchanged after addition of 4-hydroxytamoxifen (Fig. 5 compare lanes 4 and 5).

Plakophilin-1/ER Initiates Desmosome Formation in A431D Cells

We next examined the effect of plakophilin-1/ER on desmosome formation in A431D cells. Previously, others have shown that desmoplakin accumulation into punctate structures is indicative of desmosomal plaque assembly [Bornslaeger et al., 1996]. Treatment of A431D or A431DN cells with 4-hydroxytamoxifen did not alter the diffuse cytoplasmic localization of desmoplakin (Fig. 6A,B,E,F,G). In contrast, desmoplakin localization in A431D cells expressing plakophilin-1/ER following 4-hydroxytamoxifen treatment revealed a punctate cytoplasmic pattern (Fig. 6D). When A431DN cells expressing plakophilin-1/ER were treated with 4-hydroxytamoxifen desmoplakin rapidly redistributed to a punctate staining pattern in the cytosol as well at cell borders (Fig. 6I and J). Desmoplakin staining after 24 h treatment with 4-hydroxytamoxifen remained punctate and staining was seen in the cytosol as well as at cell borders. These data suggest that plakophilin-1 is capable of initiating the assembly of desmosomal plaque proteins but a classical cadherin is required for cell border localization of the desmosomal plaque.

To verify that cells form desmosomes in the presence of 4-hydroxytamoxifen we examined A431DN cells expressing plakophilin-1/ER using electron microscopy. A431D cells and A431DN cells fail to form desmosomes [Lewis et al., 1997] regardless of 4-hydroxytamoxifen treatment. A431DN cells expressing plakophilin-1/ER do not assemble electron dense desmosomal structures in the absence of 4-hydroxytamoxifen (Fig. 7A). However, following the addition of 4-hydroxytamoxifen to the culture media for 24 h numerous desmosomes were observed between adjacent cells (Fig. 7B and C). Attempts to observe desmosomes in A431D cells expressing plakophilin-1/ER were unsuccessful. In the absence of a classical cadherin, desmosomal components appear to co-localize at the plasma membrane (see below)

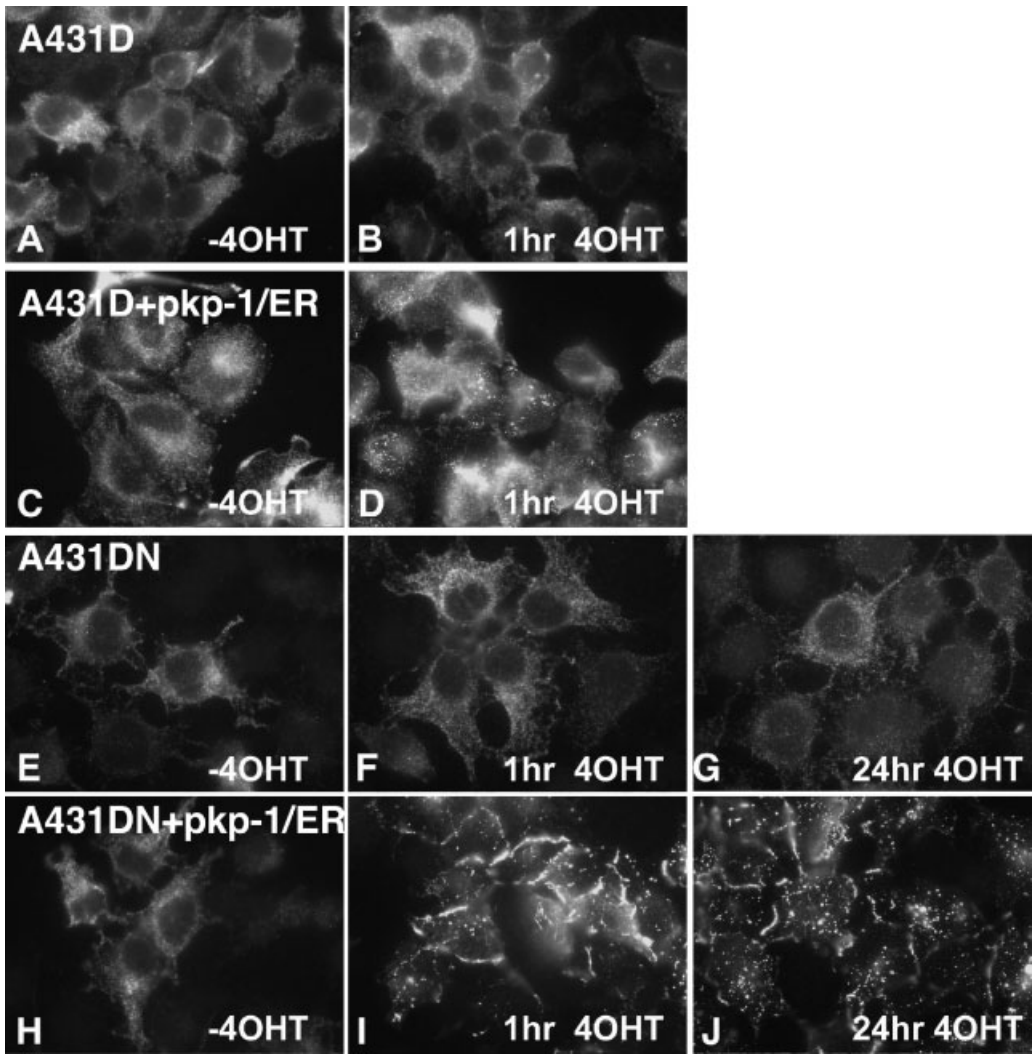

Fig. 6. Activation of plakophilin-1/ER leads to redistribution of desmoplakin. Untreated A431D cells (**panel A**), A431D cells treated with 100 nM 4-hydroxytamoxifen for 1 h (**panel B**), untreated A431D cells expressing plakophilin-1/ER (**panel C**), or A431D cells expressing plakophilin-1/ER treated with 100 nM 4-hydroxytamoxifen for 1 h (**panel D**) were grown on glass coverslips and processed for immunolocalization of desmoplakin. Note the punctate cytoplasmic localization of desmoplakin in panel D. Untreated A431DN cells (**panel E**), A431DN cells treated with 100 nM 4-hydroxytamoxifen for 1 h (**panel F**) or 24 h (**panel G**), untreated A431DN cells expressing plakophilin-1/ER (**panel H**), or A431DN cells expressing plakophilin-1/ER treated with 100 nM 4-hydroxytamoxifen for 1 h (**panel I**) or 24 h (**panel J**) were grown on glass coverslips and processed for immunolocalization of desmoplakin.

however we were unable to identify electron dense desmosomal plaques in these cells.

It is possible that the punctate structures we observed in A431D cells expressing plakophilin-1/ER upon addition of 4-hydroxytamoxifen were not actually at the cell surface and may be present in cytoplasmic vesicles. To verify the presence of these desmosomal structures at the plasma membrane we utilized immunofluorescence microscopy of permeabilized and non-permeabilized plakophilin-1/ER expressing A431D cells using an antibody directed against the extracellular domain of desmoglein 2. Following the addition of 4-hydroxytamoxifen to these cultures desmoglein 2 co-localized with desmoplakin in punctate structures (Fig. 8A–C) in permeabilized cells. Staining of non-permeabilized cells also revealed punctate localization of desmoglein 2 (Fig. 8D–F) similar to those seen in permeabilized cells suggesting that the desmoplakin positive structures observed following 4-hydroxytamoxifen addition were complexed with desmosomal cadherins at the cell surface. Desmoplakin was

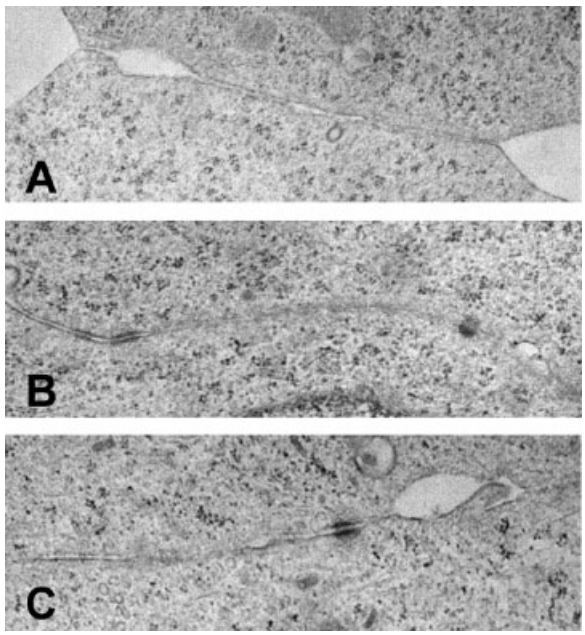

Fig. 7. Ultrastructural examination of desmosomes in A431DN cells expressing plakophilin-1/ER. Confluent A431DN cells expressing plakophilin-1/ER in the absence (**A**) or presence (**B** and **C**) of 100 nM 4-hydroxytamoxifien for 24 h were processed for electron microscopy. In the absence of 4-hydroxytamoxifen, no electron dense desmosomal plaques were observed while these structures were readily detected in cells treated with 4-hydroxytamoxifen.

also observed in these punctate structures co-localized with desmocollin 2, plakophilin-2, and plakophilin-3 (data not shown). However in the absence of a classical cadherin the plasma membrane associated punctate complexes are unable to localize to sites of cell–cell contact.

Interestingly, desmosome assembly in A431DN cultures appears to be a focal event suggesting that some desmosomal component is limiting in these cultures. A431DN clones were chosen based on N-cadherin expression and relatively high desmoplakin expression. However, even in these A431DN clones desmoplakin expression is heterogeneous. In general, cells with the highest levels of desmoplakin are more likely to display punctate desmoplakin staining following 4-hydroxytamoxifen treatment.

Time Course of Desmosome Organization in A431DN Cells Expressing Plakophilin-1/ER

The aforementioned experiments clearly show that we can induce the formation of desmosomes in A431DN cells expressing plakophilin-1/ER by adding 4-hydroxytamoxifen. To further characterize desmosome organization we performed a time course experiment. Desmoplakin redistribution was observed as early as 10 min following the addition of 4-hydroxytamoxifen (Fig. 9D). Following 20 min of 4-hydroxytamoxifen treatment, desmoplakin was

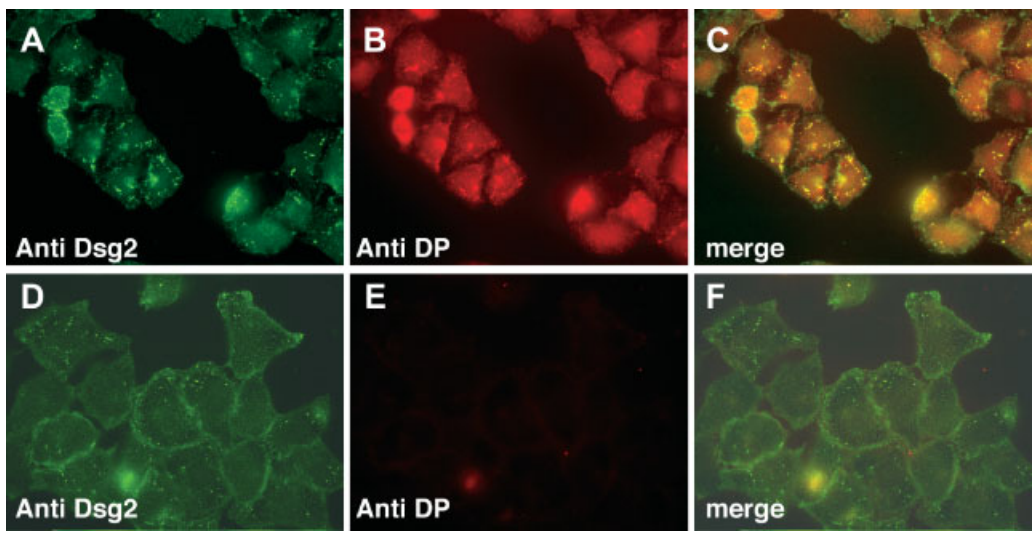

Fig. 8. Desmoglein 2 and desmoplakin co-localize at the plasma membrane following addition of 4-hydroxytamoxifen. A431D cells expressing plakophilin-1/ER were treated with 100 nM 4-hydroxytamoxifen overnight and processed for immunofluorescence microscopy. Cells were fixed using 1% paraformaldehyde and co-localization of desmoglein 2 and desmoplakin was assessed in cells permeabilized using 0.2% triton X-100 (**panels A–C**) or in non-permeabilized cells (**panels D–F**). Note the co-localization of desmoglein 2 and desmoplakin in permeabilized cells and the lack of desmoplakin signal in non-permeabilized cells.

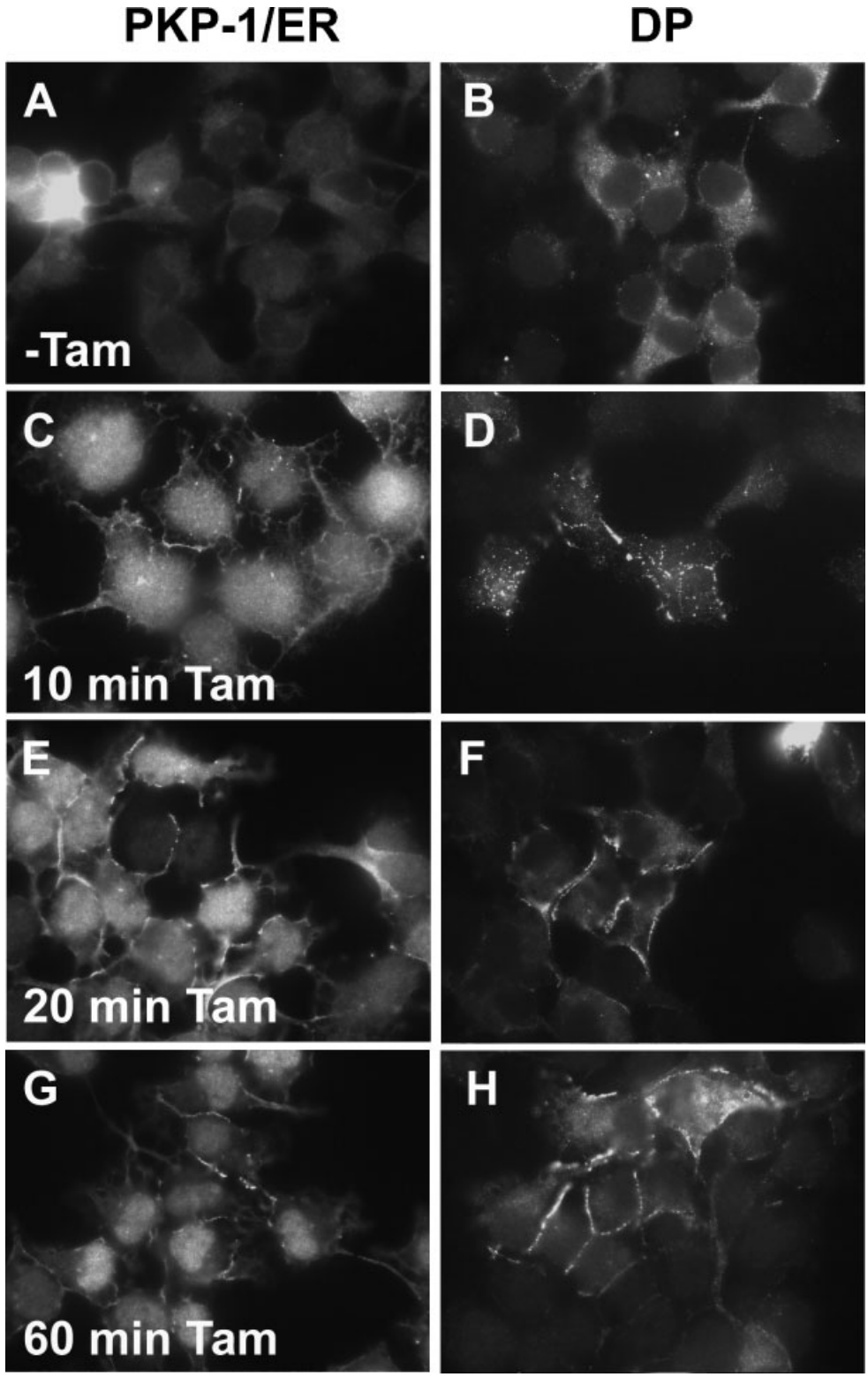

Fig. 9. Time course of desmoplakin redistribution in A431DN cells expressing plakophilin-1/ER. A431DN cells expressing plakophilin-1/ER were cultured on glass coverslips and treated with 100 nM 4-hydroxytamoxifen for the indicated times and the distribution of plakophilin-1/ER (**panels A**, **C**, **E**, and **G**) and desmoplakin (**panels B**, **D**, **F**, and **H**) were visualized. Punctate desmoplakin immunostaining can be observed as early as 10 min following the addition of 4-hydroxtamoxifen.

completely redistributed to cell borders and did not change following 60 min of treatment.

Concomitant with desmosome organization observed by immunofluorescence we expected that desmosomal components would also be incorporated into an NP-40 insoluble pool. To test this, we compared the solubility of desmosomal components following 4-hydroxytamoxifen addition in A431DN/neo cells and in A431DN cells expressing plakophilin-1/ER. In A431DN cells expressing plakophilin-1/ER the desmosomal cadherins shifted to the NP-40 insoluble pool following one hour of 4-hydroxytamoxifen treatment (Fig. 10 compare lanes 5 and 6 to lanes 7 and 8). In A431DN/neo cells, the solubility of the desmosomal cadherins did not change, suggesting that 4-hydroxytamoxifen treatment alone does not influence desmosomal cadherin solubility (Fig. 10 compare lanes 1 and 2 with lanes 3 and 4). Interestingly, the solubility of plakoglobin did not change following 4-hydroxytamoxifen treatment. The solubility of plakophilin-1/ER was slightly shifted to the NP-40 insoluble pool. This could be due to incorporation into the desmosomal plaque or transport to the nucleus. Studies of the incorporation of plakophilin-1/ER into the nucleus

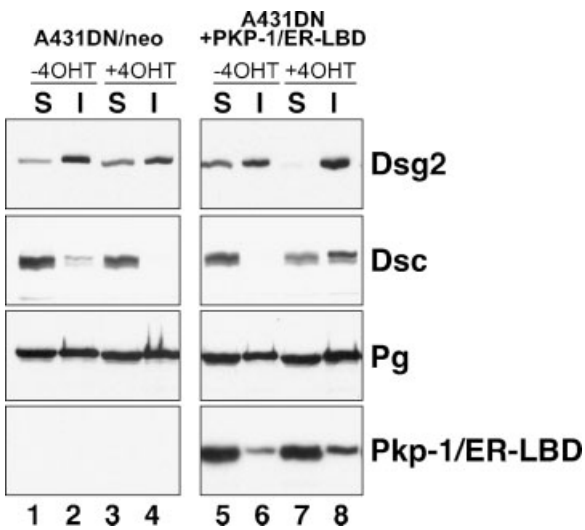

Fig. 10. NP-40 solubility of desmosomal components following 4-hydroxytamoxifen addition. NP-40 soluble (**lanes 1**, **3**, **5**, and **7**) and NP-40 insoluble (**lanes 2**, **4**, **6**, and **8**) cell lysates were prepared from A431DN/neo (lanes 1–4) cells and A431DN cells expressing plakophilin-1/ER (lanes 5–8) with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) 100 nM 4-hydroxytamoxifen treatment. Equal protein from each cell lysate was resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted using antibodies specific for desmoglein 2 (Dsg2), desmocollin (Dsc), plakoglobin (Pg), and plakophilin-1 (Pkp-1/ER).

are currently under way. Taken together, the experiments presented here clearly show that plakophilin-1/ER activation by 4-hydroxytamoxifen leads to desmosome assembly in A431DN cells.

DISCUSSION

In the present study, we have developed a unique cell culture system to evaluate the contribution of plakophilin-1 to desmosome assembly. As expected, the exogenously expressed plakophilin-1/ER localized to cell–cell borders and the nucleus following the addition of 4-hydroxytamoxifen. In addition, incorporation of plakophilin-1/ER did not perturb endogenous desmosome organization in A431 cells. Therefore, our plakophilin-1/ER system allows us to rapidly redistribute plakophilin-1/ER in cells expressing the fusion protein using 4-hydroxytamoxifen.

Previous studies aimed at understanding desmosome assembly have relied on culture systems in which desmosome assembly is induced by raising extracellular calcium (calcium switch) or by transiently transfecting multiple desmosomal components [Bornslaeger et al., 2001; Burdett and Sullivan, 2002; Koeser et al., 2003]. A drawback to such studies is the multitude of effects a calcium switch has on cells, limiting our ability to carefully dissect the mechanisms of desmosome assembly. A431D cells are an ideal system for studying desmosome assembly due to the loss of classical cadherin expression, which prevents assembly of both adherens junctions and desmosomes. Re-expression of a classical cadherin alone restores the ability of the cells to form adherens junctions, but does not restore desmosome assembly [Lewis et al., 1997]. Previously, overexpression of plakoglobin and E-cadherin was shown to be sufficient to induce desmosome assembly in A431D cells. Under these conditions desmosome formation was a rare event and we were unable to isolate clonal cell-lines that reliably assembled desmosomes. In the present study we show that activation of plakophilin-1/ER by addition of 4-hydroxytamoxifen can initiate desmosome formation in A431DN cells. These data suggest that plakophilin-1 may provide an additional mechanism that cells can use to assemble desmosomes. A puzzling aspect to our system is the fact that A431DN cells express plakophilin-2 and plakophilin-3 endogenously and fail to assemble

desmosomes. Expression and activation of plakophilin-1/ER in A431DN cells restores desmosome assembly. These experiments suggest that the endogenous plakophilins are incapable of desmosome assembly. This may simply be due to decreased expression or some uncharacterized post-translational event that leads to inactivation of the endogenous plakophilins. Overexpression of plakophilin-3 in A431DN cells also results in desmosome assembly (data not shown) therefore desmosome assembly is not unique to plakophilin-1 in this system.

Desmosome assembly in A431D and A431DN cells expressing plakophilin-1/ER occurs in roughly 80% of the cells suggesting that there is some limiting desmosomal component, possibly desmoplakin. Immunofluorescence localization of various desmosomal components revealed homogeneous expression of desmogleins, desmocollins, plakoglobin, plakophilin-2, and plakophilin-3 (data not shown). However desmoplakin expression is often heterogenous in A431D cells and clonal lines derived from A431D cells (A431DN cells). Expression of plakophilin-1/ER in A431DN cells allows us to effectively synchronize desmosome assembly by adding 4-hydroxytamoxifen. This allows us to examine desmosome assembly in a population of cells over a relatively short period of time. In addition, the retrovirally expressed plakophilin-1/ER fusion protein is stably maintained in A431 and A431D cells thus eliminating variability due to transient expression systems.

In A431D cells expressing plakophilin-1/ER, desmosomal plaque complexes assemble at the cell surface. However, these complexes are unable to localize to sites of cell–cell contact and therefore cannot generate functional cell–cell adhesion. Attempts to identify desmosomal plaques at the plasma membrane in A431D cells expressing plakophilin-1/ER were unsuccessful. When a classical cadherin is present (A431DN cells) the desmosomal structures localize to cell–cell borders where one would expect an adhesive complex to be localized. These data suggest that in A431D cells a classical cadherin is not required for assembly of the desmosomal plaque but rather is involved in targeting the desmosomal components to sites of cell–cell contact. In these studies we used the classical cadherin N-cadherin because we were able to generate stable cell-lines expressing this cadherin and the expression levels were maintained over long periods in culture. The ability of E-cadherin to direct desmosome localization has also been tested. In these experiments, E-cadherin was as effective as N-cadherin in directing desmosome localization to cell–cell borders (data not shown). These data would suggest that the classical cadherins are interchangeable in directing desmosome assembly to sites of cell–cell adhesion in our A431D cell system.

In an attempt to identify the domains of plakophilin-1 that are responsible for desmoplakin redistribution we constructed ER fusion proteins containing the plakophilin-1 head domain alone or the arm repeat domain alone and analyzed the ability of these fusion proteins to assemble desmosomal complexes. Previous reports have identified the plakophilin-1 head domain as the site of interaction with desmoplakin, desmoglein, and keratin intermediate filaments [Kowalczyk et al., 1999; Hatzfeld et al., 2000]. Therefore, we hypothesized that this domain may be sufficient to direct desmosome assembly. Interestingly, neither of these fusion proteins was capable of directing desmosomal plaque formation in A431D or A431DN cells. This suggests that the arm repeat domain also plays a role in desmosome assembly. However, proteins that interact directly with the arm repeat domain of plakophilin-1 have not been identified. Hatzfeld et al. [2000] have suggested that the arm repeat domain interacts with the actin cytoskeleton based on co-localization experiments in transiently transfected HaCat and Hela cells. However, during the course of our studies we have not observed co-localization of plakophilin-1 with the actin cytoskeleton in A431 cells.

Previously, plakophilin-1 loss has been identified as the cause of ectodermal dysplasia/skin fragility syndrome [McGrath et al., 1997]. Patients with truncating mutations in plakophilin-1 exhibited various skin defects and desmosomes were very small and few in numbers. From these observations it has been suggested that plakophilin-1 is important for assembly and stabilization of the desmosomal plaque. However, from our experiments we suspect that there may be more than one mechanism to initiate desmosome assembly. Patients lacking plakophilin-1 have desmosomes although they are small in size and few in number. In addition, various cell-lines of simple epithelial origin form desmosomes in the absence of plakophilin-1. Our parental A431

cells do not express detectable levels of plakophilin-1 but these cells make numerous desmosomes in culture. Our studies would suggest that plakophilin-1 participates in one mechanism of desmosome assembly and cells lacking expression of plakophilin-1 initiate formation of desmosomes using a separate mechanism, possibly utilizing other plakophilin family members. Recently others have suggested that plakophilin-2 is important for the formation of the desmosomal plaque in HT1080 cells in the absence of plakophilin-1 [Koeser et al., 2003]. These data suggest that plakophilins may play a central role in directing desmosome assembly.

In conclusion, we have developed a cell culture system in which we can synchronize desmosome formation in A431D cells by activating plakophilin-1/ER. Our data, together with that in the literature, suggest that there are multiple mechanisms to initiate desmosome assembly, one of which is dependent on plakophilin-1. A431D cells expressing plakophilin-1/ER provide a unique cell culture system in which we can initiate assembly of the desmosomal plaque in the absence of adherens junctions, providing a model system to dissect the mechanism of plakophilin-1 mediated desmosome assembly.

ACKNOWLEDGMENTS

We thank Dr. Pierre Chambon for the estrogen hormone binding domain cDNA, Dr. Al Reynolds for the LZRS retroviral expression system, and Dr. Kathleen Green for anti-desmoplakin serum.

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