Desmoplakin assembly dynamics in four dimensions: multiple phases differentially regulated by intermediate filaments and actin

Lisa M. Godsel,1 Sherry N. Hsieh,1 Evangeline V. Amargo,1 Amanda E. Bass,1 Lauren T. Pascoe-McGillicuddy,1,4 Arthur C. Huen,1 Meghan E. Thorne,1 Claire A. Gaudry,1 Jung K. Park,1 Kyunghee Myung,3 Robert D. Goldman,3,4 Teng-Leong Chew,3 and Kathleen J. Green1,2

1Department of Pathology, 2Department of Dermatology, 3Department of Cell and Molecular Biology, and 4The R.H. Lurie Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

The intermediate filament (IF)–binding protein desmoplakin (DP) is essential for desmosome function and tissue integrity, but its role in junction assembly is poorly understood. Using time-lapse imaging, we show that cell–cell contact triggers three temporally overlapping phases of DP-GFP dynamics: (1) the de novo appearance of punctate fluorescence at new contact zones after as little as 3 min; (2) the coalescence of DP and the armadillo protein plakophilin 2 into discrete cytoplasmic particles after as little as 15 min; and (3) the cytochalasin-sensitive translocation of cytoplasmic particles to maturing borders, with kinetics ranging from 0.002 to 0.04 μm/s. DP mutants that abrogate or enhance association with IFs exhibit delayed incorporation into junctions, altering particle trajectory or increasing particle pause times, respectively. Our data are consistent with the idea that DP assembles into nascent junctions from both diffusible and particulate pools in a temporally overlapping series of events triggered by cell–cell contact and regulated by actin and DP–IF interactions.

Introduction

Desmosomes are intercellular adhesive junctions that anchor the intermediate filament (IF) cytoskeleton to the plasma membrane. A critical role for these junctions in resisting the forces of mechanical stress is supported by the existence of autoimmune and inherited desmosome diseases that result in skin and heart fragility (for review see Godsel et al., 2004). Desmosomes must also be dynamic because their remodeling is essential in wound healing, development, and morphogenesis. However, the mechanisms that regulate the dissolution and assembly of intercellular junctions during these processes are not well understood.

In desmosomes, transmembrane members of the cadherin family, the desmogleins (Dsgs) and desmocollins (Dscs), cooperate to form the adhesive interface (Garrod et al., 2002). Their cytoplasmic tails associate with armadillo proteins, plakoglobin (Pg), and plakophilins (PKPs 1–3; Schmidt and Jager, 2005). These cytoplasmic plaque proteins interact with the IF-binding protein desmoplakin (DP), which anchors stress-bearing IFs to the desmosomal plaque (for review see Godsel et al., 2004).

DP is an essential component of desmosomes. Severing DP’s connection with IFs impairs cell–cell adhesive strength in vitro (Bornslaeger et al., 1996; Huen et al., 2002). Furthermore, DP mutations or loss of DP results in skin and heart defects in humans and mice (Armstrong et al., 1999; Whittock et al., 1999; Norgett et al., 2000; Gallicano et al., 2001; Vasioukhin et al., 2001; Alcalai et al., 2003; Rampazzo et al., 2003; Jonkman et al., 2005; for review see Godsel et al., 2004). DP is a member of the plakin family of cytolinkers with an NH2-terminal plakin domain, which is important for association with the junctional plaque through interactions with Pg and PKPs, and a central α-helical rod domain that is important for homodimerization (for reviews see Godsel et al., 2004; Jefferson et al., 2004). The COOH terminus comprises three plakin repeat domains, a specialized linker, and terminal regulatory regions that cooperate to facilitate association with IFs (Stappenbeck et al., 1993; Kouklis et al., 1994; Meng et al., 1997; Smith and Fuchs, 1998; Choi et al., 2002; Fontao et al., 2003).
Previous studies tracked the redistribution and stabilization of desmosomal cadherins and plaque components in response to increased extracellular calcium (Watt et al., 1984; Jones and Goldman, 1985; Mattey and Garrod, 1986a; Penn et al., 1987; Pasdar et al., 1991). Collectively, these studies suggested that desmosomal cadherins and DP exist in separate compartments and are integrated into junctions at the cell surface. However, the existence and nature of cytoplasmic precursors that contain DP is controversial. Some authors reported that DP-containing particles associated with IFs become redistributed to sites of cell–cell contact upon shifting cells from low to normal calcium, paralleling a reduction of cytoplasmic particles (Jones and Goldman, 1985; Pasdar and Nelson, 1988a,b; Pasdar et al., 1991). These observations led authors to hypothesize that the DP particles are desmosomal precursors. However, direct evidence supporting this idea is lacking, and other investigators have suggested that the particles are desmosomal remnants targeted for degradation (Mattey and Garrod, 1986a,b; Duden and Franke, 1988).

Another unresolved question is how the cytoskeleton might regulate desmosome assembly and DP recruitment to contact sites. Some investigators suggested that DP may be delivered to developing contacts on IFs (Jones and Goldman, 1985), whereas others concluded that IFs are not required (Baribault and Oshima, 1991; Bornslaeger et al., 1996; Vasioukhin et al., 2001). Phosphorylation of DP at Ser2849 was shown to impair interactions between the DP COOH terminus and IFs (Stappenbeck et al., 1994; Meng et al., 1997; Fontao et al., 2003); however, its role in DP localization during desmosome assembly is unknown. Likewise, the role of microfilaments in desmosome assembly is poorly understood. Although one study suggested that cytochalasin B inhibits desmosome assembly (Inohara et al., 1990), another suggested that microfilaments are not required for the accumulation of desmosome components but are for their proper organization and stability at the plasma membrane (Pasdar and Li, 1993).

To overcome the limitations inherent in establishing a temporal sequence of events from fixed specimens, we have used GFP-tagged DP to follow the assembly and fate of desmosome precursors in living cells during junction assembly. Our findings support the idea that cell–cell contact triggers a temporally coordinated process beginning with an initial, rapid local assembly phase followed by the assembly and translocation of cytoplasmic particles containing DP and its armadillo protein binding–partner PKP2. DP dynamics during junction assembly are regulated by both DP–IF interactions and actin microfilaments. These mechanisms are likely to play key roles in coordinating desmosome assembly and maturation during the epithelial remodeling that occurs in development and wound healing.

Results

DP-GFP incorporates normally into desmosomes and is present in discrete cytoplasmic particles

To establish the temporal sequence of DP dynamics and fate during desmosome assembly we generated three COOH-terminal GFP constructs: full-length DP (DP-GFP), a phosphorylation point mutant (DPgly-GFP) with a Ser→Gly replacement at position 2849, and a truncated DP encompassing the NH2-terminal 584 amino acids (DPNTP-GFP; Fig. 1 A). DP-GFP, DPgly-GFP, and DPNTP-GFP were expressed at the predicted molecular weights in inducible A431 (Fig. 1 B).
and transient SCC9 (Fig. 1 C) transfectants. Full-length GFP-tagged proteins were expressed at only 1/7 to 1/13 of the level of endogenous DP. DPNTP was present at 1/4 of the level of endogenous DP. Furthermore, the expression level (Fig. 1, B and C) and localization (see Fig. 3) of other desmosomal proteins were not detectably altered. DP-GFP was present in discrete cytoplasmic dots, similar to those previously reported for endogenous DP (Figs. 2–5), and during junction assembly DP-GFP accumulated at borders with a time course comparable to that reported for endogenous protein, where it colocalized in a
DP-GFP participates in three temporally overlapping phases of DP dynamics that are triggered by cell-cell contact

To directly examine DP dynamics during desmosome assembly and to address whether DP-containing particles are desmosome precursors, we used time-lapse imaging to follow the fate of DP-GFP in living cells during the process of cell–cell contact formation. In the representative experiments shown in Fig. 2 (A and B), A431 cells inducibly expressing DP-GFP were subjected to light scrape wounding, and z-stacks were collected over time to observe de novo desmosome formation.

Three temporally overlapping phases of DP dynamics were observed (Fig. 2 A, a). First, small fluorescent puncta appeared along the forming border as early as 3 min after cell–cell contact and underwent coalescence and accretion over time (Fig. 2, A [b] and B [green arrows]; and Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1). Second, new DP-GFP particles formed in the cytoplasm near newly contacting borders as early as 15 min after cell–cell contact (Fig. 2, A [c and d] and B, arrows; and Videos 1–3, arrows). Third, a subset of preexisting and newly formed particles translocated in an anterograde direction to new contact sites (Fig. 2 A [d] and B; and Videos 1–3). Occasionally, we observed another type of dynamics in which linearly arrayed dots appeared to stream toward remodeling borders (Video 1). Our quantitative analysis focused on particle behavior associated with newly forming contacts (Fig. 2, A [d] and B, yellow arrows; and Videos 2 and 3, yellow arrows). Particles moved with variable kinetics and instantaneous velocities ranging from 0.002 to 0.04 μm/s. The observed dynamics were independent of differences in DP-GFP expression level, cell background, or clonal variability.

Quantification of fluorescence at newly forming borders over time revealed two waves of increasing intensity that were present in >78% of the 32 borders analyzed (Fig. 2, C and D). The first wave, corresponding to phase I (Fig. 2, C and D, green arrow), was characterized by an ~30–45 min period of increasing fluorescence. The formation of new cytoplasmic particles (phase II; Fig. 2, C and D, red arrow) was first observed at the beginning or during the plateau that followed. The second wave of intensity corresponded with the onset of particle translocation to borders (phase III; Fig. 2, C and D, yellow arrow). Together, these data support a model in which cell–cell contact triggers three temporally overlapping phases of DP dynamics. These include two productive events that lead to an increase in border fluorescence intensity: a local assembly phase at the membrane, followed by the incorporation of cytoplasmic DP particles.

Assembly-competent DP particles are associated with the armadillo protein PKP2

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Toward defining the biochemical nature of DP-containing precursors and the relationship of particle composition to their dynamic behaviors, we performed light and EM analysis of cells undergoing junction assembly. Immunofluorescence analysis of cells after a calcium switch revealed that PKP2 colocalized prominently with particles near nascent junctions (Fig. 3). Two other armadillo family members, PKP3 and Pg, were present in some particles, but not concentrated to the extent of PKP2 (Fig. 3 B). Some larger cytoplasmic structures colocalized with the cadherin Dsc2, but were typically more perinuclear and likely represented engulfed desmosomes. Furthermore, DP-GFP particles close to the zone of cell contact did not colocalize with an intracellular membrane dye, supporting the idea that the precursors are not membrane-bound (Fig. 3 C).

Conventional EM revealed the presence of electron dense particles, similar to those previously reported, associated with IFs and aligned with microfilament bundles (Fig. 4, A and C, arrows). During early stages of assembly, immunogold analysis of whole mount material revealed DP-GFP at cell–cell interfaces in the absence of well-formed plaques and in the cytoplasm close to the zone of contact (Fig. 4, B, D, and G). DP-GFP also localized to desmosomes later in the assembly process (Fig. 4 F). In the cytoplasm, DP-GFP was in clusters of varying sizes and was often associated with IF bundles (Fig. 4, B, D, and F–H). PKP2 also appeared at borders early (Fig. 4 G),
later in desmosomes, and in cytoplasmic particles of varying size that colocalized with DP (Fig. 4, G and H). Single or sparsely organized IFs inserted into DP-GFP–positive early contacts (Fig. 4 G, bottom right inset), whereas larger bundles associated with more mature plaques (Fig. 4 F).

To directly correlate particle composition with behavior during junction assembly, we performed retrospective analysis. All phase II DP-GFP particles that appeared during imaging, including those that began to move toward the developing intercellular borders, contained PKP2 (Fig. 5 A and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1), but not Dsc2 (Fig. 5 D and Video 6). Although Pg (Fig. 5 C) and PKP3 (Fig. 5 B and Video 5) were seen in some particles, neither were concentrated to the extent of PKP2 nor was translocation dependent on their presence. In fact, many PKP3- or Dsc2-positive particles were present before cell contact (Fig. 5, B [blue circle] and D [pink circle]) and some moved in a retrograde (Fig. 5 B, orange and yellow ovals) or random (Fig. 5 D, blue circle) motion. Many particles formed in close association with the keratin IF cytoskeleton (Fig. 5 E), although it was not possible to conclude that all particles formed on IFs. Collectively, these studies demonstrate that DP-GFP selectively assembles with PKP2 into precursor particles during cell contact–initiated desmosome assembly.

Uncoupling DP from IFs or interfering with microfilament organization alters DP particle movement

To test whether loss of the IF-binding domain alters DP dynamics, time-lapse imaging of DPPTP-GFP was performed in cells at the leading edge of a scrape wound. DPPTP-GFP was present in cytoplasmic particles with a wider size range than those assembled from wild-type DP. Furthermore, although ~70% of DP-GFP particles colocalized with keratin IFs, only ~30% of DPPTP-GFP particles colocalized with IF (Fig. 6 A).

Fluorescent DPPTP-GFP puncta appeared at the forming border within 5–10 min of cell–cell contact and underwent coa-
Figure 5. **DP-GFP and PKP2 colocalize in the assembly-competent particles that appear after cell–cell contact.** Wounded DP-GFP expressing A431 monolayers were imaged at 1-min intervals, fixed, and processed for immunofluorescence after cell–cell contact. (A) PKP2 localizes in all cytoplasmic particles that appeared and moved toward the forming border. Representative images illustrate the fates of numbered DP-GFP particles. Merge shows extensive DP and PKP2 colocalization (Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1). (B) Most phase II particles that moved toward the forming border did not contain PKP3 (Video 5). However, many preexisting particles (blue circle) and particles moving in a retrograde fashion (orange and yellow ovals) did colocalize with PKP3. (C) Most phase II particles including those that moved toward the forming border did not contain Pg. (D) Most phase II particles did not contain Dsc2 (Video 6). Dsc2-containing particles were larger, perinuclear, exhibited random movements, and were often present before contact (pink and blue circles). (E) Many phase II DP-GFP containing particles appeared to be associated with IF. Bar, 10 μm.
Figure 6. **DPNTP-GFP exhibits altered dynamics and delayed junction assembly.** (A) DPNTP-GFP does not colocalize with the keratin IF network. DP-GFP or DPNTP-GFP expressing A431 cells (green) were fixed and stained for keratin (red). Although DP-GFP particles aligned with keratin IF, DPNTP-GFP particles were distributed more randomly (see merged and enlarged regions, right). Bar, 10 μm. (B) Wounded DPNTP-GFP expressing A431 monolayers were imaged at 5-min intervals (Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1). DPNTP-GFP phase I was comparable to DP-GFP with fluorescence appearing within 5–10 min of contact. Yellow, red, and green arrows follow three DPNTP-containing cytoplasmic phase III particles as they join the forming cell–cell border. Phase II was not observed. Bar, 10 μm. (C) Comparison of DPNTP-GFP (pink squares) and DP-GFP (blue diamonds) fluorescence intensity over time, from paired cells imaged under the same conditions. Although DP-GFP exhibited two waves of border intensity separated by a plateau (Fig. 2), the increase in DPNTP-GFP border fluorescence was more linear with dampened intensity. (D) Schematic depiction of DPNTP-GFP particle trajectory (left) and velocity (right) over time. Colors correspond to the yellow, red, and green arrows designating particles in B. Many particles like the one designated by the green arrow exhibited random movements, traveling a large total distance with fluctuating instantaneous velocities before incorporating. Some particles (yellow and red arrows) exhibited dynamics more similar to DP-GFP, moving directly into the borders without pausing. (E) Wounded DPNTP-GFP expressing A431 were imaged at 1-min intervals (Video 8). Phase I fluorescence appeared within 10 min of contact (green arrow). Phase III particles are marked with yellow arrows. DPNTP-GFP particles exhibited faster, more random dynamics than DP-GFP particles. Bar, 20 μm.
lescence and accretion over time (Fig. 6, B and E; and Videos 7 and 8, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1). However, the slope of increased border intensity was dampened (Fig. 6 C). Although some preexisting particles translocated to new contacts, an obvious phase II was not observed. Whereas some particles moved in a directed anterograde fashion, others exhibited random motion before translocating to cell–cell borders (Fig. 6, B, D, and E; and Videos 7 and 8). DPNTP-GFP particles exhibited more rapid movements than DP-GFP particles, reaching instantaneous velocities of 0.08 μm/s compared with 0.04 μm/s for DP-GFP particles. Thus, although IF binding is not required for DP incorporation into desmosomes, DP that lacks the IF binding site interferes with particle assembly, positioning, and dynamics.

Because DP that lacks an IF-binding domain can traffic to intercellular borders, frequently with more rapid kinetics, and IF-associated DP particles are closely associated with cortical actin (Green et al., 1987), we hypothesized that reorganization of the actin cytoskeleton might drive later phases of DP dynamics. To test this idea, we imaged DP-GFP–expressing cells over time after disrupting filamentous actin with cytochalasin D. Phase I occurred and phase II particles formed (Fig. 7 A and not depicted), but did not appear to translocate to borders (Fig. 7 A). The increase in fluorescence intensity normally associated with phase III was not observed (Fig. 7 B). These data suggest that IFs and microfilaments are both involved in regulating DP dynamics.

DP Ser2849 regulates DP’s association with IF and assembly into desmosomes

Abrogation of DP phosphorylation at Ser2849 by site-specific mutation was previously shown to enhance interactions between the

Figure 7. Maturation of DP-GFP borders is cytochalasin D sensitive. (A) Wounded DP-GFP expressing A431 monolayers were imaged at 1-min intervals in the presence of cytochalasin D. Phase I accumulation of DP-GFP (arrows) at the cell–cell border and the formation of phase II particles (arrowheads) was observed in the presence of cytochalasin D. Phase III particle translocation was not observed. (B) Fluorescence border intensity of cytochalasin D treated cells (gray squares) over time was compared with DMSO treated cells imaged in parallel (black diamonds). An initial increase in pixel intensity was observed, but did not increase appreciably over time. A second increase in border fluorescence was not observed, supporting the role of particle translocation in border maturation. Bar, 10 μm.

Figure 8. DPgly-GFP associates strongly with IF and is insensitive to forskolin-induced junction recruitment. (A) SCC9 cells were transiently transfected with both full-length DP-RFP and DPgly-GFP. Although DP-RFP was largely at cell borders, DPgly-GFP more prominently decorated IF. Bar, 20 μm. (B) Cells were fractionated into cytoplasmic (C), Triton X-100 soluble (S), and triton insoluble (I) fractions. DPgly-GFP in the insoluble fraction was 5× greater than DP-GFP. (C) SCC9 cells stably expressing DP-GFP or DPgly-GFP were treated with 100 μM forskolin for 16 h. DP-GFP fluorescence increased at cell borders and was more organized with forskolin treatment. No difference in border fluorescence intensity was observed in cells expressing DPgly-GFP. Average fluorescence intensities for a fixed cell population are depicted graphically to the right of representative fluorescence images (*, P < 0.00001). Error bars are SEM. Bar, 20 μm.
DP COOH terminus and IFs (Stappenbeck et al., 1994; Meng et al., 1997; Fontao et al., 2003). To examine the impact of this mutation on full-length DP, the distribution of transiently transfected DP-RFP and the phosphorylation-deficient mutant DPgly-GFP were compared (Fig. 8 A). DPgly-GFP coaligned extensively with IFs, with some punctate staining at borders, whereas DP-RFP was more prominent at cell borders. DP-RFP exhibited some coalignment with IFs, likely because of heterodimerization with DPgly-GFP. The ratio of DPgly-GFP in the detergent insoluble versus cytosolic pool was approximately fivefold greater than that of DP-GFP (Fig. 8 B). These data support the idea that mutation of Ser2849 enhances the association of DP with IF.

We previously demonstrated that activation of PKA using forskolin leads to the phosphorylation of Ser2849, reducing association of the DP COOH terminus with K8/18-rich IF networks (Stappenbeck et al., 1994). We hypothesized that PKA activation might release full-length DP from IFs, thus generating a larger assembly-competent pool. Supporting this idea, fluorescence intensity at borders increased twofold in forskolin-treated DP-GFP, but not DPgly-GFP cells (Fig. 8 C), without altering DP expression (not depicted), consistent with the idea that PKA promotes DP assembly into desmosomes through the phosphorylation of Ser2849. To test whether the phosphorylation-deficient mutant exhibits altered assembly...

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**Figure 9. Assembly of DPgly-GFP into desmosomes after a calcium switch is delayed.** (A) SCC9 cells transiently expressing DP-GFP or DPgly-GFP (green) were fixed at 10-min intervals after a calcium switch (0–90 min) followed by keratin staining (red). DP-GFP appeared at cell borders within 10 min and became prominent by 40 min. DPgly-GFP appeared weakly at cell borders at 20 min, with a dampened increase in intensity at 40 and 60 min. Bar, 10 μm. (B) Comparison of fluorescence intensities of borders shared by pairs of cells expressing DP-GFP or DPgly-GFP over time. DPgly-GFP exhibited significantly reduced intensities from 10–80 min (P < 0.03), normalizing at 90 min. Error bars are SEM. (C) PKP2 colocalized with DP-GFP in cytoplasmic particles and at cell borders (top) and is found in a punctate pattern along filament-associated DPgly-GFP, and at the tips of filament bundles (bottom). Bar, 5 μm.
kinetics, we calculated fluorescence intensity along contacting borders for pairs of DP-GFP– and DPgly-GFP–expressing cells after a calcium switch (Fig. 9, A and B). Between 10 and 20 min, DP-GFP began to accumulate at cell–cell contact sites, and by 40–50 min border staining was extensive. DPgly-GFP particle redistribution was delayed, with obvious fluorescence...
accumulating only after 50–60 min. By 90 min, junction staining normalized, but DPgly-GFP was also retained along cytoplasmic IFs. These results suggest that increased association with the IF cytoskeleton delays DP recruitment to cell–cell contact sites.

As shown in a previous section (Assembly-competent DP particles...), PKP2 is selectively concentrated in DP-GFP precursor particles and its association is correlated with assembly competence. To test whether PKP2’s association with DPgly-GFP is altered, we performed confocal colocalization studies. PKP2 colocalized with DPgly-GFP particles and exhibited punctate staining that was overlaid on the continuous DPgly-GFP pattern (Fig. 9 C). These data are consistent with the idea that altered association with IFs, rather than lack of PKP2 association, alters DPgly-GFP assembly kinetics.

**Discussion**

The four-dimensional analyses reported here demonstrate that during desmosome assembly DP-GFP participates in a temporally coordinated process consisting of multiple overlapping components triggered by cell–cell contact. The first response to cell contact is the rapid de novo appearance and continuing accretion of fluorescent puncta at new contact sites. DP/PKP2-positive particles then appear in the cytoplasm, some of which are subsequently transported to junctions. The initial, rapid DP accumulation at borders can occur in the absence of the later events, which are sensitive to interference with DP–IF interactions and microfilament organization.

Previous views regarding the origin and fate of cytoplasmic DP-containing particles vary. One view is that particles represent internalized membrane-bound desmosome remnants (Kartenbeck et al., 1982; Mattey and Garrod, 1986b; Duden and Franke, 1988) not used in future rounds of junction assembly (Mattey and Garrod, 1986b). We do see more perinuclear DP particles colocalizing with desmosomal cadherins, which may be engulfed desmosomes (Fig. 5). We have also observed retrograde flow of DP-GFP originating from cell–cell interfaces, perhaps similar to the irreversible uptake of fluorescent particles observed in cells expressing Dsc2-GFP (Windoffer et al., 2002). This could represent an important mechanism for down-regulating desmosome-mediated adhesion during epithelial remodeling. Those purporting that particles are not precursors have suggested that DP incorporates into desmosomes from a diffusible pool of subunits that assemble de novo at cell–cell contact sites (Duden and Franke, 1988). DPI dimers sediment at 6.7-s (O’Keefe et al., 1989) and soluble, possibly oligomeric, 7.3-s and 9-s forms have been reported in epithelial cells (Duden and Franke, 1988; Pasdar and Nelson, 1989).

Together with the data presented here, it has been shown soluble DP may provide a pool for the rapid early appearance of DP-GFP at contact sites, as well as a source of DP for phase II particle coalescence.

DP dynamics at the plasma membrane are likely coordinated with the previously reported dynamics of fluorescently labeled desmosomal cadherins (Gloushankova et al., 2003), including the aggregation of assembling Dsc2-GFP puncta (Windoffer et al., 2002). Such a scenario is consistent with immunogold EM analysis showing that Dsg3 is delivered to the plasma membrane into desmosome precursors, some of which are attached to keratin IFs (Sato et al., 2000). DP could coordinate IF attachment to nascent sites of junction assembly, an idea supported by our EM analysis showing DP-GFP associated with sparse IFs at areas of the plasma membrane early in the junction assembly process. The keratin-associated patches of Dsg3 were described as half-desmosome-like clusters, and half desmosomes were previously reported to form at free surfaces in the absence of cell contact (Demlehner et al., 1995; Sato et al., 2000). Although we cannot rule out this type of assembly in our studies, the majority of plasma membrane–associated DP appeared to be at sites of intercellular contact.

Our work also provides compelling evidence to support the model that DP particles are nonmembrane-bound desmosome precursors that become redistributed to the cell periphery after initiation of cell–cell contact (Jones and Goldman, 1985). Nonmembrane-associated DP-GFP particles formed during phase II and translocated into borders. Translocation corresponded temporally with a second increase in fluorescence intensity, which is consistent with the idea that it represents a productive phase of desmosome assembly. Retrospective analysis revealed that PKP2 was present in the assembly-competent particles, suggesting a functional requirement for PKP2 in the formation and/or translocation of these particles. Such a requirement is supported by preliminary RNA interference experiments showing that PKP2 knockdown blocks DP border accumulation (unpublished data). Consistent with the importance of PKP2 for DP plaque association, DP is uncoupled from cardiomyocyte junctions and is present as granular aggregates in the cytoplasm in mice lacking PKP2, leading to defects in heart morphogenesis (Grossmann et al., 2004). Cardiac de-
fects are also seen in humans with PKP2 mutations (Gerull et al., 2004).

The role of PKP2 in DP assembly dynamics is not yet known. PKP family members facilitate DP clustering, which may be important for the accretion of DP during phase I, as well as DP coalescence during phase II. PKP family members associate with both IFs and microfilaments (Hatzfeld et al., 2000; Hofmann et al., 2000), raising the possibility that they may coordinate interactions between DP-GFP particles and the cytoskeleton. Results using cytochalasin D and preliminary data using the myosin II inhibitor blebbistatin (unpublished data) suggest that an actomyosin-based mechanism is required for particle translocation. That the initial local assembly step is not blocked is consistent with the observation that desmosome components accumulated at the membrane of cytochalasin B–treated cells while retaining significant intracellular staining (Pasdar and Li, 1993). The speed of DP particle movement ranges from 0.002 to 0.04 μm/s, and is thus slower than that mediated by most conventional motors. DP translocation could be driven indirectly via the redistribution and contraction of the cortical actomyosin ring, known to mature in association with adherens junction formation. Actin may drive the reorganization of DP-associated IF bundles and/or chaperone DP particles to sites of assembly more directly. In the absence of the IF-binding domain, this latter mechanism may proceed more rapidly than when DP is tethered to IF. Consistent with this, instantaneous DPNTP-GFP velocities can reach twice the speed of those observed for DP-GFP. PKP2 in DPNTP particles may coordinate association with actin, thus explaining the ability of untethered particles to reach the plasma membrane.

Although a more definitive analysis of the relationship between DP and IF during assembly awaits dual label imaging studies, our retrospective analysis suggests that many phase II dots form in close association with IF. Furthermore, compromising (DPNTP) or enhancing (DPgly) DP interactions with IFs alters the trafficking patterns and kinetics of DP incorporation into assembling desmosomes. Together, our data support the idea that cytoplasmic DP particles can act as junction precursors, but that particle translocation is only one phase of a temporally coordinated process that is regulated by the actin and IF cytoskeletons. The existence of phases with potentially different modes of assembly (e.g., diffusion for phase I not subject to inhibition by cytoskeletal drugs and an actomyosin-dependent mechanism for phase III) could help to reconcile the differing models of desmosome assembly. Regulation of each of these steps is likely to play key roles in regulating the efficiency of desmosome formation during epithelial remodeling that occurs in development and wound healing.

Materials and methods

Generation of constructs

Full-length human DP (DP-GFP; p804) containing the entire coding region (1–8,613 nucleotides) fused to GFP at the COOH terminus was generated by cloning DP into pEGFP-N1 (CLONTECH Laboratories, Inc.), resulting in plasmid p928. To generate the plasmid for use in the doxycycline (DOX)-inducible A431 system, the DP full-length coding region including EGFP was excised from p928 and subcloned into the pTRE2 response plasmid to generate p926. A red fluorescent chimera of DP containing the entire coding region of 1–8,613 nucleotides fused to DsRed2 at the COOH terminus was generated by cloning DP from p928 in DsRed2-N1 (CLONTECH Laboratories, Inc.), resulting in p991. Full-length DP containing a single Ser→Gly substitution at position 2849 of the protein (DPgly-GFP) was generated by replacing a COOH-terminal fragment in p804 with the same fragment from p86, which harbors a single nucleotide change at position 8,403 to generate p906. To generate the plasmid for use in the Tet-On A431 system (CLONTECH Laboratories, Inc.), the DP-coding region, including EGFP, was excised from p906 and subcloned into the pTRE2 response plasmid to generate p927. Cloning steps for DPNTP-GFP and DPgly-GFP in the pTRE2 vector are detailed in a previous publication (Huen et al., 2002).

Cell lines, culture conditions, and transfections

SCC9 cell lines (a gift from J. Rheinwald, Harvard Medical School, Boston, MA) were maintained in DME/F-12, 10% FBS, and 1% penicillin/streptomycin. Stable cell lines were maintained in the same culture, with the addition of 400 μg/ml G418. A431-inducible lines were maintained in DME, 10% FBS, 1% penicillin/streptomycin, 400 μg/ml G418, and 1 μg/ml puromycin. Protein expression was induced by culturing the cell lines in 1–4 μg/ml DOX for 24 h. Transient transfections were performed on cultures grown on Type I collagen–coated coverslips (0.1 mg/ml collagen I diluted in 0.02 sodium acetate acid for 1 h at room temperature using the manufacturer’s protocol; BD Biosciences) using ExGen 500 according to the manufacturer’s protocol (Fermentas Life Sciences). Generation of pTREOn DPNTP-GFP A431 cells was described previously (Huen et al., 2002). DP-GFP and DPgly-GFP were generated as described for DPNTP-GFP cells, screened for expression after 1–4 μg/ml DOX treatment for 24 h, and analyzed by direct fluorescence and immunoblotting. SCC9 stable lines were generated by transfecting cells with either DP-GFP or DPgly-GFP DNA using ExGen 500 transfection reagent (Fermentas Life Sciences) according to the manufacturer’s protocol, selected with 400 μg G418, ring cloned, and screened by direct fluorescence and immunoblotting.
Antibodies and reagents
The following primary antibodies were used: Rabbit polyclonals NW161 against the DP NH2 terminus (Bornslaeger et al., 1996), an anti-EGFP antibody (CLONTECH Laboratories, Inc.), and anti-GAPDH (Novus Biologicals); mouse monoclonals 7G6 against Dsc3, 6D8 against Dsg2, 11E4 against Fg (gifts from M. Wheelock, K. Johnson, and D. Johnson, University of Nebraska), SCC9 (gift from P.M. Green, Omaha, NE), KSB17.2 against keratin 18 (Sigma-Aldrich), MAAB6135 against PKP2 a and b (Marine Biotechnology Services); 23E3 against PKP3 (gift from F. van Roy, VIB-Ghent University, Ghent, Belgium; Bonnie et al., 2003), hVIN1 against vinculin (Sigma-Aldrich), JL8 against EGFP (CLONTECH Laboratories, Inc.), and a guinea pig polyclonal against PKP2 (gift from W. W. Franke, German Cancer Research Center, Heidelberg, Germany). The following secondary antibodies were used: Alexa Fluor 568 goat anti–mouse IgG at 1:300 (Invitrogen), HRP-conju- gated goat anti–mouse and goat anti–rabbit IgG at 1:5000 (KPL, Inc.); and 10- or 18-nm colloidal gold-conjugated goat anti–rabbit, 10-nm gold-conjugated goat anti–mouse (Sigma-Aldrich), and 18-nm gold-conju- gated donkey anti–guinea pig IgG (Jackson ImmunoResearch Labora- tories) at 1:5. The Image-iT LIVE intracellular membrane labeling kit (Invitrogen) was used to stain membranes after fixation following the manufacturer’s instructions.

Preparation of cell lysates and immunoblot analysis
Whole cell lysates in Laemmli sample buffer were resolved by 5 or 7.5% SDS-PAGE, and immunoblotted as previously described (Angst et al., 1990), and immunoreactive proteins were visualized using enhanced chemiluminescence. Sequential detergent extractions were performed as described (Palka and Green, 1997). JL8 (1:1,000), hVIN1 (1:1,000), 11E4 (1:1,000), 6D8 (1:500), MAAB6013 (straight supernatant), KSB17.2 (1:1,000), anti-GAPDH (1:2,000), and NW161 (1:5,000).

Immunofluorescence analysis and image acquisition
Cells were seeded onto 40-mm-diam glass coverslips and Lab-Tek chambered coverglass slides (Nunc) coated with 0.1 ng/ml collagen I, as described in Cell lines, culture conditions, and transfections, and grown to confluence. Monolayers were wounded with small, random holes using a 26-gauge needle and placed at 37°C for 60 min before mounting coverslips into the FCS2 live-cell imaging chamber (Bioptechs, Inc.) or placing chambered slides directly onto the stage. The cell chamber was filled with imaging media (Hanks balanced salt solution, 20 mM Hepes, 1% FBS, 2 mM l-glutamine, 4.5 g/L glucose, 1x amino acids; recipe courtesy of G. Kreitzer, Weill Medical College of Cornell University, New York, NY). Fluorescence and differential interference contrast or phase time-lapse recordings were obtained at 63× (PL APO, NA 1.3) using mercury or hala- gen excitation with an inverted microscope (model DMIRE2, Leica). Imaging was performed with an Orca 100 CCD camera and OpenLab 2.2.5 software or the Application Solution Multidimensional Workstation (ASMDW; Leica) at consistent time intervals of 15 s to 5 min. Images were processed using Photoshop 6.0 and assembled using Adobe Premiere or ASMDW software and MetaMorph 6.1 imaging software (Universal Imaging Corp.). The ASMDW contains a DMIRE2 inverted microscope fitted with a Coolscan HI (Roper Scientific) camera, a high-precision scanning stage for simulta- neous collection of several fields, and a 63× (HCX PL APO, glycerine, NA 1.3) objective fitted with a piezo element for rapid collection of z-stacks (13–15; 0.4–0.5-μm stacks at 10 μm intervals). For time-lapse microscopy, monolayers were incubated on coverslips and incubated with 2–4% polyethylene glycol. Gelsolin was used to remove actin and mi- crystals during junction assembly

Calcium switch and forskolin and cytochalasin D treatments
SCC9 cells expressing GFP-tagged DP proteins or primary mouse kerati- nocytes were incubated in low calcium medium (DME with 0.05 mM CaCl2) for 16 h, switched to normal growth media containing ~1.2 mM Ca2+ to induce cell junction assembly for time periods ranging up to 3 h, and processed for immunofluorescence or ultrastructural analysis. For forsk-olin experiments, N-Bars stable lines were incubated with 100 μM for- skolin (Sigma-Aldrich) for 16 h at 37°C. For cytochalasin D time-lapse im- aging experiments, wounded monolayers were incubated with 0.3 μg/ml cytochalasin (Calbiochem) or the DMSO carrier and imaged for 230 min.

Time-lapse imaging and retrospective immunofluorescence of DP-GFP particles during junction assembly
Cells were seeded onto 40-mm-diam glass coverslips or Lab-Tek chambered coverslip slides (Nunc) coated with 0.1 ng/ml collagen I, as described in Cell lines, culture conditions, and transfections, and grown to confluence. Monolayers were wounded with small, random holes using a 26-gauge needle and placed at 37°C for 60 min before mounting coverslips into the FCS2 live-cell imaging chamber (Bioptechs, Inc.) or placing chambered slides directly onto the stage. The cell chamber was filled with imaging media (Hanks balanced salt solution, 20 mM Hepes, 1% FBS, 2 mM l-glu- tanes, and 1 mM MgCl2, and 1 mM EGTA) containing 1% Triton X-100 and 4% polyethylene glycol. Gelsolin was used to remove actin and mi- crystals during junction assembly

Fluorescence intensity of cytoplasmic particles and cell–cell borders, particle kinetics, and densitometric analysis of immunoblots
Fluorescence pixel intensity at cell borders over time was determined by multiplying the average pixel intensity per cell by the area of the defined border divided by the border length. Background intensity was subtracted from background intensity. Error bars represent standard error. Quantitative analysis and statistical analysis was performed using t test. Particle velocity and dis- tance traveled was calculated for DP containing cytoplasmic particles. Average relative fluorescence intensity was determined by dividing inte- grated pixel intensity of particles in the cytoplasm by integrated pixel in- tensity of an identically sized region of fluorescence intensity at the cell–cell border. All calculations were performed using OpenLab 2.2.5 or MetaMorph 6.1 imaging software. Densitometric analyses were per- formed by scanning immunoblots using the UMAX 1100 and Magic-Scan32 v4.5 software and analyzed using Molecular Analyzer software (Bio-Rad Laboratories).

Ultrastructural analysis
Conventional EM of primary mouse keratinocytes was performed as de- scribed previously (Starger et al., 1978; Jones and Goldman, 1985; Green et al., 1987). Immunogold labeling was performed for EM using cytoskeletal preparations of SCC9 cells constitutively expressing DP-GFP or DPGlyGFP as previously described (Svitkina and Borisy, 1998) with the following exceptions. Cells were lysed in PEM buffer (100 mM Pipes, pH 6.9, 1 mM MgCl2, and 1 mM EGTA) containing 1% Triton X-100 and 4% polyethylene glycol. Gelsolin was used to remove actin and mi- crystals were not preserved. Preparations were examined with a transmission electron microscope (model 100CX, JEOL). Negatives were scanned using the UMAX 1100 and MagicScan32 v4.5 (UMax Data Systems, Inc.) software and processed using Photoshop CS and illustrates

9.0.1 or CS (Adobe).

Online supplemental material
Videos 1 and 2 correspond to Fig. 2 A, and Video 3 corresponds to Fig. 2 B. Videos 4, 5, and 6 correspond to Fig. 5 A, B, and D, respectively. For Videos 1–6, A431 cells were induced to express DP-GFP with DOX. Vir- uses 1 and 2 correspond to Fig. 6 B and E, respectively, in which A431 cells were induced to express DPNTP-GFP. Videos 9 and 10 correspond to Fig. 10 A and D, respectively, in which A431 cells were induced to express DPGlyGFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1.

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