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


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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 (Barbault 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-tagged DP constructs: wild-type DP (DP-GFP), a phosphorylation point mutant (DPgly-GFP) with a Ser→Gly substitution at residue 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 SCC9 cells (Fig. 1 C).

Figure 1. GFP-tagged DP expression and localization. (A) GFP-tagged proteins: full-length DP (DP-GFP) with a wild-type Ser residue at position 2849, a phosphorylation point mutant (DPgly-GFP) with a Ser→Gly replacement at position 2849, and a mutant lacking the central rod and IF-binding domain (DPNTP-GFP). (B) A431 cells were treated with DOX to induce DP-GFP, DPgly-GFP, and DPNTP-GFP, which were expressed at 1/7, 1/13, or 1/4 the level of endogenous DP, respectively. GFP-tagged protein expression did not affect Dsg2, Pg, and PKP2. (C) GFP-tagged and endogenous DP from transiently expressing SCC9 cells were separated on 5 or 7.5% gels. Full-length GFP-tagged proteins (ζGFP) were expressed at ≤1/5 the level of endogenous DP. Steady-state levels of endogenous DP, Dsg2, Pg, or PKP2 were not affected compared with parental SCC9s (−). Loading control: vinculin.

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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

Figure 2. DP accumulation at newly contacting cell-cell borders occurs in three phases. (A) Wounded DP-GFP-expressing A431 monolayers were imaged at 1.5-min intervals. (a) Three phases of DP-GFP dynamics were observed after cell-cell contact. In phase I, fluorescence began to accumulate at sites of contact within ~3–10 min. In phase II, cytoplasmic particles began to appear at 30–45 min of contact. In phase III, cytoplasmic particles moved toward the maturing border. To the right of the schematic are images of the microscopic field at 0 and 60 min after contact. Colored boxes indicate enlarged regions (b–d; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1). Bar, 20 μm. (b) Phase I: DP appeared within 7 min of cell-cell contact. (c) Phase II: DP dots appeared de novo in the cytoplasm within 25 min of contact. (d) Phase III: Cytoplasmic DP dots moved in an anterograde direction and incorporated into the forming border (Video 2). (B) Wounded DP-GFP expressing A431 monolayers were imaged at 1-min intervals (Video 3). The green arrow highlights DP that accumulated within 9 min of cell contact (phase I). Cytoplasmic dots appeared upon initiation of cell contact (49 and 64 min; phase II) and translocated to borders (yellow arrows; phase III). Bar, 10 μm. Fluorescence intensity over time was calculated for representative borders from the videos in A and B (C) and from 32 other movies (D). In the graphs two waves of border fluorescence corresponded with the onset of phases I (green arrows) and III (yellow arrows). Phase II particle formation occurred at the beginning of or during the plateau between phases I and III (red arrows). Results are representative of data obtained from >50 movies.
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

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 DPNTP-GFP was performed in cells at the leading edge of a scrape wound. DPNTP-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 DPNTP-GFP particles colocalized with IF (Fig. 6 A).

Fluorescent DPNTP-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
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 coaligns 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

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.

**DPgly-GFP exhibits altered dynamics and increased pause times during translocation to maturing cell contacts.** Analysis of DPgly-GFP by four-dimensional imaging revealed that the average onset of phase I was delayed and fluorescence intensity suppressed, as compared with DP-GFP in cells with continuous staining along IFs (Fig. 10, A and B) and cells with more particulate staining (not depicted). Phase II was either absent or delayed, with phase II particles appearing on DP-coated filaments, which most likely represented keratin bundles (Fig. 10 A and Video 9, available at http://www.jcb.org/cgi/content/full/jcb.200510038/DC1). Phase III DPgly-GFP particles exhibited more halting movements and greater pause times than DP-GFP particles (Fig. 10, A, C, and D; and Videos 9 and 10), although instantaneous velocities for DPgly-GFP particles still fell within the range of those exhibited by DP-GFP. A representative particle from Fig. 10 A was stationary from one time point to the next 73% of the time (Fig. 10 C), compared with 33% for a DP-GFP particle from a paired movie (not depicted). 85% of DPgly-GFP particles exhibited pause times, whereas only 30% of DP-GFP particles paused. Collectively, these data suggest that either impairing or enhancing DP–IF interactions delays DP’s recruitment to desmosomes.

**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 association with IFs is not required for DP incorporation, but can regulate the course of this process. It is interesting to note that DP particle speeds fall into the same range reported for anterograde keratin particle and keratin “squiggle” movements in keratinocytes (Helfand et al., 2004). Our previous studies suggested that ectopically expressed DP forms oligomeric structures containing IF poly peptides that resemble the desmosomal plaque (Stappenbeck and Green, 1992). Therefore, it seems possible that DP-keratin oligomers may be translocating together to cell–cell borders.

We demonstrate that mutation of Ser2849 leads to an increase in the detergent-insoluble cytoskeletal fraction of DP, consistent with previous findings that PKA-dependent phosphorylation regulates DP–IF binding (Stappenbeck et al., 1994). The delay in phase II could thus be explained by a reduction in the availability of diffusible DP due to its sequestration on IF. However, other mechanisms, such as alterations in binding to proteins required for the assembly process, cannot be ruled out. DPgly particles also exhibited greater pause times during translocation, suggesting that junction maturation is altered.

In normal physiological situations, it is probable that desmosome precursor particles comprise a mixture of phosphorylated and unphosphorylated DP. Likewise, due to heterodimerization, it is likely that DPgly-GFP particles contain some endogenous protein. In this way, DPgly-GFP shifts the balance to the less phosphorylated state, allowing us to detect the functional impact of low levels of Ser2849 phosphorylation; i.e., delayed DP recruitment into junctions. Thus Ser2849 phosphorylation may act as a rheostat, regulating precursor recruitment into the plaque, indirectly regulating the rate at which strengthening of desmosome adhesion occurs.

Collectively, these results provide compelling support for 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 pEFGP-C1 (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 mRFP1 at the COOH terminus was generated by cloning DP from p928 into pDsRed2-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 p96, 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 plasmid 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 Tet-On 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/ml G418, ring cloned, and screened by direct fluorescence and immunoblotting.
Cells incubated with MAB6013 or 23E3 were extracted with 0.5% Triton X-11E4 (1:200), 23E3 (1:100), and MAB6013 (undiluted supernatant). Immunofluorescence using KSB17.2 (1:200), 7G6 (1:200), 6D8 (1:50), and 11E4 against Pg (gifts from M. Wheelock, K. Johnson, and J. Wahl, University of Nebraska Medical Center, Omaha, NE), KSB17.2 against keratin 18 (Sigma-Aldrich), MAAB6013 against PKP2 a and b (Marine Biotechnology Services), 23E3 against PKP3 (gift from F. van Roy, VIB-Ghent University, Ghent, Belgium; Bonne et al., 2003), hVIN-1 against vinculin (Sigma-Aldrich), JLB-8 against EGF (CLONTECH Laboratories, Inc.), and a guinea pig polyclonal against PKP3 (gift from W. K. Hall, University of Nebraska Medical Center, Omaha, NE), and an anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at 1:5. The Image-T LIVE intracellular membrane labeling kit (In-vitrogen) 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). JLB (1:1,000), hVIN-1 (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 cover slips or LabTek chambered coverglass slides (Nunc) coated with 0.1 mg/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 cover slips 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 halogen illumination with an inverted microscope (model DMIRE2; Leica) fitted 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 CoolSNAP HQ (Roper Scientific) camera, a high-precision scanning stage for simultaneous 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 per time point) in a 37°C climate chamber. For retrospective analysis, monolayers were fixed at various times after the observation of cell–cell contact during time lapse imaging and indirect immunofluorescence analysis performed as described in Immunofluorescence analysis and image acquisition. Images were relocated and z-stacks captured with the ASMDW at 63×. All images obtained with the ASMDW were processed and deconvoluted using a blind deconvolution synthetic algorithm, and z-stacks from each time point were assembled into multi-image projections using system software.

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 field by the area of the defined border divided by the border length. Background intensity was subtracted from border intensity. Error bars represent standard errors. Immunoblots were scanned using the UMAX 1100 and MagicScan32 v.4.5 software and analyzed using Molecular Analyst software (Bio-Rad Laboratories).
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