Adducin Is Required for Desmosomal Cohesion in Keratinocytes*

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Vera Rötzer1, Andreas Breit5, Jens Waschke11, and Volker Spindler12

From the1Institute of Anatomy and Cell Biology, Ludwig-Maximilians-Universität, Munich D-80336 and the5Walther-Straub-Institute for Pharmacology and Toxicology, Ludwig-Maximilians-Universität, Munich D-80336, Germany

Adducin is a protein organizing the cortical actin cytoskeleton and a target of RhoA and PKC signaling. However, the role for intercellular cohesion is unknown. We found that adducin silencing induced disruption of the actin cytoskeleton, reduced intercellular adhesion of human keratinocytes, and decreased the levels of the desmosomal adhesion molecule desmoglein (Dsg)3 by reducing its membrane incorporation. Because loss of cell cohesion and Dsg3 depletion is observed in the autoantibody-mediated blistering skin disease pemphigus vulgaris (PV), we applied antibody fractions of PV patients. A rapid phosphorylation of adducin at serine 726 was detected in response to these autoantibodies. To mechanistically link autoantibody binding and adducin phosphorylation, we evaluated the role of several disease-relevant signaling molecules. Adducin phosphorylation at serine 726 was dependent on Ca2+ influx and PKC but occurred independent of p38 MAPK and PKA. Adducin phosphorylation is protective, because phosphorylation-deficient mutants resulted in loss of cell cohesion and Dsg3 fragmentation. Thus, PKC elicits both positive and negative effects on cell adhesion, since its contribution to cell dissociation in pemphigus is well established. We additionally evaluated the effect of RhoA on adducin phosphorylation because RhoA activation was shown to block pemphigus autoantibody-induced cell dissociation. Our data demonstrate that the protective effect of RhoA activation was dependent on the presence of adducin and its phosphorylation at serine 726. These experiments provide novel mechanisms for regulation of desmosomal adhesion by RhoA- and PKC-mediated adducin phosphorylation in keratinocytes.

Adducins are a family of actin-binding proteins comprised of three isoforms (α, β, and γ). The α- and γ-isoforms are ubiquitously expressed in most tissues, whereas β-adducin is most abundant in erythrocytes and brain (1). Adducin is the major component of the spectrin-based membrane cytoskeleton, thereby recruiting spectrin to actin filaments and promoting the assembly of a spectrin-actin meshwork underneath the plasma membrane (2, 3). Besides mediating the association between spectrin and F-actin, adducin is indispensable for the regulation of actin filaments via bundling and capping of the fast-growing end of actin filaments (4, 5). Moreover, it is well established that adducin localizes to cell-cell contacts in vitro (6) and in vivo (7) suggesting a relevance of adducin in proper assembly of F-actin bundles at intercellular junctions (8). The interaction of adducin with spectrin and actin is regulated by Ca2+ and calmodulin (4, 9) and through phosphorylation by various protein kinases such as protein kinase A (PKA), protein kinase C (PKC) (6, 10), and Rho-kinase (11, 12). It is noteworthy that Rho-GTPases are both important for actin cytoskeleton regulation (13–15) and are involved in signaling induced by autoantibodies in pemphigus (16–18).

Pemphigus vulgaris (PV)3 is an autoimmune disease of the skin caused by autoantibodies directed against the adhesion molecules desmoglein (Dsg) 1 and 3 (19). There is growing evidence that both direct inhibition of Dsg3 interaction by antibody binding as well as intracellular signaling are necessary for intraepidermal blister formation (20). It has been shown that loss of keratinocyte cohesion in response to PV-IgG was accompanied by profound alterations of the cortical actin belt including fragmentation of actin filament bundles and increased stress fiber formation (21, 22). Pharmacological inhibition of p38 MAPK (21) or activation of RhoA (16–18) was sufficient to block the PV-IgG-mediated loss of cell adhesion as well as effects on actin cytoskeleton reorganization. Moreover, for RhoA-mediated protection against autoantibody-induced loss of cell cohesion a requirement of cortical actin polymeri-

3 The abbreviations used are: PV, pemphigus vulgaris; RhoA, Ras homolog gene family, member A; Dsg, desmoglein; DP, desmoplakin; CK, cytokera-

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1To whom correspondence may be addressed: Pettenkoferstrasse 11, D-80336 Munich, Germany. Tel.: 49-89-2180-7260; Fax: 49-89-2180-72602; E-mail: jens.waschke@med.uni-muenchen.de.
2To whom correspondence may be addressed: Pettenkoferstrasse 11, D-80336 Munich, Germany. Tel: 49-89-2180-7261; Fax: 49-89-2180-72602; E-mail: volker.spindler@med.uni-muenchen.de.

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Adducin and Keratinocyte Cohesion

zation has been demonstrated (23). Concomitantly, a recent study reported a direct association of Dsg3 with actin and its involvement in actin dynamics (24).

In the first part of the present study, we focused on the role of adducin for desmosomal keratinocyte cohesion and on the turnover of Dsg3. Because we identified adducin to become phosphorylated in response to pemphigus autoantibodies, we tested the contribution of several known PV-relevant signaling molecules for adducin phosphorylation in the second part of the study.

EXPERIMENTAL PROCEDURES

Cell Culture and Test Reagents—The spontaneously immortalized human keratinocyte cell line HaCaT was grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 50 units/ml of penicillin, and 50 μg/ml of streptomycin (both AppliChem, Darmstadt, Germany), and maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. For all experiments 1 × 10⁶ cells/cm² were seeded and grown in uncoated 24-well plates (Greiner Bio-One, Kremsmuenster, Austria) to confluence in high calcium medium (1.8 mM CaCl₂ 2H₂O) within 4 days.

The PV serum was drawn from a patient with active PV suffering from both oral and skin lesions. The ELISA values (Euroimmun, Luebeck, Germany) were 11,550 (Dsg3) and 375 (Dsg1) units/ml. Purification of IgG was performed as described previously (22), and IgG fractions were applied at 0.5 mg/ml. AK23, a monoclonal pathogenic antibody derived from a pemphigus mouse model (25), was purchased from Biozol, Eching, Germany, and used at 75 μg/ml. A IgG fraction of a healthy volunteer was applied at 0.5 mg/ml. Escherichia coli cytotoxic necrotizing factor (CNF)-1 (activation of RhoA, Rac1 and Cdc42) and Yersinia pseudotuberculosis CNFγ (activation of RhoA) were purchased from Cytoskeleton (Denver, CO) and preincubated at a dose of 1 μM for 6 h. Rho-kinase was effectively blocked by y27632 (Merck, Darmstadt, Germany) at a dose of 1 mM for 6 h. p38 MAPK inhibitor SB202190 (Merck) was used at 30 μM for 30 min or 24 h, respectively. The PKC activator phorbol 12-myristate 13-acetate (PMA) (Sigma) was applied at 50 nM, the inhibitor BIM-X (Enzo Life Sciences, New York, NY) was used at 0.5 mg/ml, and both were preincubated for 30 min. The PKA specific inhibitor H-89 (Sigma) was used at 10 μM for 4 h. The PKC activator forskolin was used at 30 μM for 30 min or 24 h, respectively. The PKC activator phorbol 12-myristate 13-acetate (PMA) (Sigma) was applied at 50 nM, the inhibitor BIM-X (Enzo Life Sciences, New York, NY) was used at 0.5 mg/ml, and both were preincubated for 30 min. The PKA specific inhibitor H-89 (Sigma) was used at 10 μM and preincubated for 2 h. BAPTA-AM (Sigma), a cell-permeable Ca²⁺ chelator, was applied at 50 μM for 4 h.

Transfection—Adducin-specific small-interfering RNA (α-adducin siRNA: L-009487-00-0005 or γ-adducin siRNA: L-008468-00-0005) was purchased from Thermo Fisher Scientific/Dharmacon, Lafayette, IN. The plasmids pEGFP-C1-α-adducin, pEGFP-C1-α-adducin (S726A), pEGFP-C1-α-adducin (S716A/S726A), and pEGFP-C1-α-adducin (S726D) were kindly provided by Hong-Chen Chen (National Chung Hsing University, Taichung, Taiwan) (26). The plasmid pEGFP-N1-Dsg3 was a generous gift by Yasushi Hanakawa (Ehime University School of Medicine, Japan). For all experiments, HaCaT cells were cultured to 70–80% confluence in 24-well plates or in μ-slide 8-well imaging chambers (Ibidi, Martinsried, Germany) within 24 h and transfected with TurboFect™ (Fermen-
Adducin and Keratinocyte Cohesion

Rac and Rho Protein Activation Assay—To test the pharmacological activators CNF-1 or CNFy and the effects of AK23 or PV-IgG on activation of Rac and RhoA, HaCaTs were grown on 24-well plates and treated with the respective substances before being subjected to G-LISA® Rac or RhoA activity assays (Cytokeleton). Assays were applied following the manufacturer’s protocol and the optical density was read with a plate reader device at 490 nm (TECAN, Maennedorf, Switzerland).

Image Analyses—The intensity of cortical actin was evaluated in ImageJ as arbitrary units by drawing a rectangle with a fixed size over the membrane in each quadrant of a cell double labeled for F-actin and α- or γ-adducin and background intensity measured on an uncovered area of the slide was subtracted. Then the four intensity values obtained per cell and color were averaged and plotted as dot plot with the x axis representing α- or γ-adducin and F-actin shown on the y axis. Thus, each data point represents a single cell.

The fragmentation index was calculated by measuring the length of the cell circumference and the length of the cortical F-actin underneath the membrane. F-actin length was divided by the length of the cell circumference, yielding the percentage of the membrane that was covered by F-actin in a given cell. Analysis was carried out in cells transfected with n.t.-siRNA and cells were transfected with either α- or γ-adducin siRNA. In the latter two cases, we also distinguished between cells with clear knockdown and cells without effective knockdown. This was possible because these two cell populations were clearly distinguishable in the respective adducin staining.

The width of Dsg3 staining on the membrane was quantified by measuring the length of Dsg3 perpendicular to the cell membrane in each quadrant of a cell. The resulting values were averaged yielding a mean value of Dsg3 width per cell.

Statistics—Error bars represent mean ± S.E. Data for two groups were compared using two-tailed Student’s t test. For multiple group comparisons, analysis of variance was performed followed by Bonferroni post hoc test. Statistical significance was assumed when \( p < 0.05 \).

RESULTS

Adducin Silencing Promoted Cell Dissociation and Reduced Desmosomal Dsg3 Protein Content—In fully confluent HaCaT, both α- and γ-adducin were expressed in comparable amounts as detected on mRNA (Fig. 1, A and B) and protein level (Fig. 1C) and colocalized extensively with the cortical actin belt (Fig. 1D). Under conditions of effective silencing via siRNA (Fig. 1, B and C), both adducin isoforms were absent in large areas of the monolayer (Fig. 1D). In these areas, the cortical actin belt appeared strongly disorganized and fragmented (arrows) compared with cells unaffected by siRNA transfection. This is also reflected in the image analysis by which the cortical F-actin intensity was reduced under conditions of decreased adducin staining intensity (Fig. 1E). Here, each data point represents a single cell, the blue colored points denote cells with clearly positive knockdown, the purple spots are cells in which apparently the knockdown was not effective. The F-actin intensity in cells with visible knockdown was significantly reduced in comparison to non-target controls or cells unaffected by adducin targeting. An evaluation of cortical actin fragmentation was car-
Adducin and Keratinocyte Cohesion

Figure A

378 bp
251 bp
137 bp

Figure B

251 bp
137 bp

Figure C

α-adducin
γ-adducin
α-tubulin

Figure D

n.t. siRNA
α-adducin siRNA
n.t. siRNA
γ-adducin siRNA

Figure E

F-actin intensity [AU] vs. α-adducin intensity [AU]

Figure F

F-actin intensity [AU] vs. γ-adducin intensity [AU]

Figure G

F-actin intensity [AU] vs. γ-adducin intensity [AU]
Adducin silencing promotes cell dissociation and reduces desmosomal Dsg3 levels. A, after siRNA-mediated silencing of the respective adducin isoform, confluent monolayers were subjected to dispase-based dissociation assays. Specific adducin knockdown resulted in a significant increase of fragment numbers compared with controls and silencing of both adducin isoforms led to further loss of cell adhesion (N = 7, n > 14, *p < 0.05 versus control). B, representative images of monolayer fragmentation that were taken immediately after assay performance. C, immunoblot analysis demonstrated a decrease of Dsg3 but not of E-Cadherin protein levels after isoform-specific knockdown of adducin (N = 5, *p < 0.05 versus control). D, Triton X-100 separation of cell lysates displayed a specific depletion of Dsg3 in the cytoskeleton-associated fraction after adducin knockdown. DP was used to indicate the desmosomal pool.

We next measured cell cohesion of HaCaT keratinocytes after adducin knockdown using dispase-based dissociation assays. Control knockdown cells were largely resistant to mechanical stress (fragment numbers: 1.1 ± 0.1), whereas silencing of either α- or γ-adducin increased fragment numbers to 18.1 ± 2.4 and 18.3 ± 1.4, respectively (Fig. 2, A and B). Silencing of both α- and γ-adducin led to a further increase of cell dissociation (29.4 ± 2.9 fragments).

Previously, adducins were demonstrated to be involved in adherens and tight junction remodeling in SK-CO15 cells (8). Because our studies demonstrated loss of cell adhesion following adducin silencing, we investigated the expression of desmosomal and adherens junction molecules. After gene silencing of either adducin isoform, the amount of Dsg3, a desmosomal adhesion molecule highly relevant for keratinocyte cell adhesion (27), was significantly decreased to 60.5 ± 8.9 (α-adducin knockdown) or 63.1 ± 5.1% (γ-adducin knockdown) compared with controls (Fig. 2C). This effect seemed to be restricted to desmosomes, as the adherens junction molecule E-cadherin was not significantly affected by adducin silencing (E-cadherin protein level: 93.2 ± 6.1 and 92.1 ± 8.2%, respectively). Because the effects of silencing of either α- or γ-adducin were identical, we further focused on α-adducin only. To examine whether Dsg3 was depleted from desmosomes or from non-cytoskeleton-associated pools, we performed Triton X-100 extraction of HaCaT lysates after α-adducin knockdown. In contrast to the non-cytoskeleton-bound fraction (105.8 ± 5.6%), a decrease of Dsg3 levels to 69.0 ± 5.1% (α-adducin knockdown) was significantly decreased to 60.5 ± 8.9 (α-adducin knockdown) or 63.1 ± 5.1% (γ-adducin knockdown) compared with controls (Fig. 2C).

Desmoplakin (DP) served as marker for the desmosomal pool. GAPDH indicated the non-cytoskeletal fraction. Interestingly, a partial colocalization of DP and α-adducin was visible by immunostaining (Fig. 2E, arrows).
Adducin and Keratinocyte Cohesion

The effects of α-adducin silencing on adhesion molecules were also reproduced by immunolocalization studies. To indicate cells with effective knockdown, α-adducin siRNA was labeled (Fig. 3A, arrows). α-Adducin knockdown was also paralleled by reduction of β-spectrin, a known binding partner of adducins (Fig. 3B). Dsg3 staining appeared reduced in intensity and was more diffusely distributed at the cell membrane in cells with effective α-adducin siRNA uptake (Fig. 3B). However, in line with the Western blot experiments, this effect was not observed for E-cadherin localization (Fig. 3C). Similarly, no changes in distribution of the desmosomal plaque protein DP or intermediate filament system (marked by a pan-cytokeratin antibody) were detectable in α-adducin siRNA-transfected cells (Fig. 3F and G). Insets, ×2.5 magnification of areas indicated. Scale bar, 20 μm, N = 4.

FIGURE 3. Knockdown of α-adducin affects Dsg3 and β-spectrin distribution in HaCaTs. A, for immunolocalization studies Cy3-labeled siRNA (red) was used. Effective α-adducin silencing was proven by immunostaining against α-adducin (green, arrows; left two panels) and in parallel by Western blot analysis (right panel). B, in cells with effective α-adducin knockdown, a reduction of β-spectrin staining was detectable. C, compared with control knockdown, cells with effective adducin silencing displayed reduced and diffusely distributed Dsg3 staining, whereas the localization of E-cadherin (D) and the tight junction molecule claudin-1 (E) was unaffected. Similarly, no changes in distribution of the desmosomal plaque protein DP or intermediate filament system (marked by a pan-cytokeratin antibody) were detectable in α-adducin siRNA-transfected cells (F and G). Insets, ×2.5 magnification of areas indicated. Scale bar, 20 μm, N = 4.

Adducin Silencing Delays Membrane Incorporation of Dsg3—Our data indicate that α-adducin regulates the turnover of Dsg3 in keratinocytes. We thus sought to investigate by which mechanisms adducin regulates Dsg3 content. We first tested whether α-adducin silencing promoted the internalization of Dsg3. However, colocalization of Dsg3 with the early endosome marker EEA-1 was a rather rare event under control knockdown conditions and was not increased after α-adducin silencing (Fig. 4, A and B, arrows). Therefore, we tested the other possibility that adducin is involved in membrane incorporation of Dsg3 and/or lateral mobility in the membrane, the latter of which may be necessary for incorporation into desmosomes (29). To test this hypothesis, we utilized FRAP. HaCaT cells were transfected with GFP-Dsg3 and either α-adducin or non-target siRNA (Fig. 4C). Interestingly, in α-adducin knockdown cells GFP-Dsg3 intensity at the membrane was reduced compared with controls (Fig. 4D). Furthermore, fluorescence recovery of GFP-Dsg3 in areas that were bleached by high power laser excitation was significantly reduced in cells with α-adducin silencing over the time of measurement (Fig. 4, E and F). These data suggest that adducin primarily regulates Dsg3 membrane incorporation and/or lateral mobility.

Pemphigus Autoantibodies and RhoA Induce Rapid Phosphorylation of Adducin—Given our finding that adducin silencing led to depletion of Dsg3, an event also elicited by application of pemphigus autoantibodies, we investigated the effects of an IgG fraction of PV patients (PV-IgG) on adducin localization. Incubation with PV-IgG for 24 h resulted in fragmentation of addu-
Adducin staining at the membrane (Fig. 5A). This phenomenon was observed primarily at sites where intercellular gaps formed (arrows). Nevertheless, actin colocalization was preserved.

Next, we analyzed adducin phosphorylation and protein levels in response to pemphigus autoantibodies. We applied PV-IgG (Fig. 5B, middle panel), or AK23, a pathogenic monoclonal antibody derived from a pemphigus mouse model (Fig. 5B, lower panel) to confluent HaCaT cells at time points ranging from 5 min to 24 h. No changes in $\alpha$-adducin levels were detectable throughout this time frame (Fig. 5B). However, compared with incubation with control-IgG (Fig. 5B, upper panel), phosphorylation at Ser-726 was significantly increased after 10 min of PV-IgG incubation to $171.2 \pm 11.9\%$ and after AK23 incubation to $184.6 \pm 16.7\%$. Similar effects were obtained for $\gamma$-adducin (data not shown). Collectively, these data demonstrate rapid adducin phosphorylation in response to pemphigus autoantibodies.

To investigate the mechanisms by which pemphigus autoantibodies lead to phosphorylation of adducin, we tested several pathways that are known to be altered in pemphigus. We here focused on RhoA, p38 MAPK, and PKC and PKA signaling.

The Protective Effect of Rho-GTPase Activation on Autoantibody-mediated Loss of Cell Cohesion Is Dependent on Adducin
Phosphorylation—Because previous data demonstrated that pharmacological activation of Rho-GTPases blocked PV-IgG-induced loss of cell adhesion and fragmentation of cortical actin (16, 17, 23), we first investigated the effects of the bacterial toxins CNF-1 (activation of RhoA, Rac1, and Cdc42) and CNFy (activation of RhoA) on phosphorylation of adducin. After testing the specificity of CNF-1 and CNFy in keratinocytes by ELISA-based assays (Fig. 5C), we treated HaCaTs with the respective compound. Interestingly, similar to AK23 and PV-IgG, both specific activation of RhoA by CNFy and activation of RhoA, Rac1, and Cdc42 by CNF-1 for 10 min (after preincubation for 6 h) led to adducin phosphorylation (Fig. 5D). Based on these data, we concluded that RhoA is the primary Rho family GTPase inducing adducin phosphorylation. In line with this, incubation of the Rho-kinase inhibitor y27632 for 1 h decreased the phosphorylation level below controls. Dispase-based assays revealed an increased number of fragments after y27632 incubation for 1 h (13.5 ± 0.8) but no alterations of cell cohesion by treatment with CNF-1 or CNFy alone (Fig. 5E). Because CNFy and CNF-1 had the same effect on adducin phosphorylation as pemphigus autoantibodies after 10 min, we tested whether these autoantibodies increase RhoA activity. However, RhoA activity was not elevated but rather reduced after 10 min (Fig. 5F), which is in line with previous data where RhoA levels were decreased after 24 h of PV-IgG incubation (16). Thus, adducin phosphorylation in response to PV-IgG incubation is independent of RhoA.

Next, we used CNFy under conditions of α-adducin silencing (Fig. 6A). In line with our previous studies, activation of Rho-GTPases was protective in control knockdowns as the AK23-induced increase of fragment numbers (20.5 ± 1.8) was blocked by co-incubation of CNFy (9.5 ± 1.0) and did not significantly differ from controls after 24 h (Fig. 6B). AK23 treatment for 24 h under conditions of α-adducin silencing further increased frag-
ment numbers (53.3 ± 6.3) compared with siRNA control. Importantly, under knockdown conditions AK23-mediated loss of cell cohesion was only partially blocked by CNFy co-incubation (fragment numbers: 43.25 ± 3.1, respectively) and still significantly differed to the respective control. Based on the finding that RhoA activation led to phosphorylation of α-adducin at Ser-726, we next applied several phospho mutants to test whether this modulates the protective effect of RhoA activation (Fig. 6C): GFP-α-adducin wild type (GFP-Addwt), GFP-α-adducin-S716A/S726A (GFP-AddmutAA), an α-adducin construct containing point mutations at Ser-716 and Ser-726 for alanine, GFP-α-adducin-S726A (GFP-AddmutA), and GFP-α-adducin-S726D (GFP-AddmutD); and D, detection of efficient transfection by Western blot analysis. E, in dissociation assays, overexpression of GFP-Addwt resulted in similar fragment numbers as GFP-controls following 24 h of AK23 incubation. AK23-mediated loss of cell adhesion was significantly blocked in these cells. In contrast, AK23-induced fragmentation was drastically increased in cells expressing GFP-AddmutAA or GFP-AddmutA compared with GFP-Addwt cells and co-treatment with CNFy was only partially protective under these conditions. Also in GFP-AddmutD-transfected cells no protective effect was observed by CNFy, indicating that this mutant had no phosphomimetic activity under these conditions (N = 6, n = 12, *, p < 0.05 versus control; #, p < 0.05 versus AK23).

FIGURE 6. The protective effect of Rho-GTPase activation is dependent on both adducin expression and phosphorylation at Ser-726. A, representative Western blot of α- or γ-adducin silencing performed in parallel to dispase-based dissociation assays. B, in dissociation studies, AK23-induced loss of cell-cell adhesion after 24 h was significantly blocked in control knockdowns by co-incubation of CNFy. Under conditions of α- or γ-adducin silencing, AK23 incubation further increased the number of fragments. In these cells, AK23 effects were not significantly blocked by co-incubation with CNFy (N = 5, n > 10, *, p < 0.05 versus control; #, p < 0.05 versus AK23).
knockdown studies, AK23-mediated cell dissociation were only partially prevented by simultaneous treatment with CNFy (GFP-AddmutAA, 43.5 ± 1.9; GFP-AddmutA, 37.75 ± 1.8 fragments). Although the results with GFP-AddmutD were similar to the other mutants and thus GFP-AddmutD had no phosphomimetic activity in this context, it still underscores the protective effect of Ser-726 phosphorylation.

To further address the role of adducin in autoantibody-mediated cell dissociation, we stained HaCaT cells expressing GFP-Addwt or GFP-Addmut after 24 h of AK23 incubation against Dsg3 (Fig. 7A). As previously reported for PV-IgG (17, 30) and AK23 (31, 32) in HaCaT and DJM-1 cells, AK23 treatment of control cells resulted in fragmentation of Dsg3 staining and formation of linear streaks perpendicular to the cell border (d–f), which led to a broadening of Dsg3 membrane staining compared with the continuously localized distribution in untreated cells (a–c). CNFy co-incubation prevented AK23-induced Dsg3 fragmentation (g–i). GFP-Addwt colocalized with Dsg3 at cell borders and was also present in the nucleus (j–l). Disruption of Dsg3 staining by AK23 incubation for 24 h was abolished in cells containing GFP-Addwt (m–o) and the parallel application of CNFy had no further effect (p–r). Dsg3 staining in both GFP-AddmutAA and GFP-AddmutA-transfected cells appeared weakened even under control conditions (s–u and ab–ad). Incubation with AK23 further increased fragmentation of Dsg3 staining (v–x and ae–ag), which was not prevented by CNFy co-incubation in both Addmut-transfected cells (y–aa and ah–aj). An evaluation of Dsg3 staining width at the membrane is provided in Fig. 7B.

Next, we investigated the effects of AK23 and CNFy on the distribution of Dsg3 in Triton X-100-soluble and -insoluble pools (Fig. 7C). AK23-mediated depletion of cytoskeletal Dsg3 was partially prevented by CNFy co-incubation in control cells and Addwt, but not in AddmutAA- or AddmutA-expressing cells. This is in line with the results from immunofluorescence analyses.

Taken together, these data indicate that adducin phosphorylation following pemphigus antibody incubation is independent of RhoA. However, the protective effect of exogenous RhoA activation on loss of cell adhesion relies on the presence of adducin as well as on its appropriate phosphorylation at Ser-726. Furthermore, adducin rendered phosphorylation-deficient at Ser-726 altered the regular Dsg3 distribution at the cell membrane.

PKC, but Not p38 MAPK or PKA Is Involved in PV-induced Adducin Phosphorylation—To elucidate by which pathway adducin is phosphorylated, we next focused on p38 MAPK and PKC.

The contribution of p38 MAPK to the pathogenesis of pemphigus vulgaris is well established (32, 33) and previous studies placed p38 MAPK upstream of RhoA in this context (16, 18). However, treatment of keratinocytes with the p38 MAPK inhibitor SB202190 had no effect on adducin phosphorylation compared with control. AK23 alone, but also the co-incubation with SB202190, led to increased adducin phosphorylation (N = 4). In Western blot analysis, alterations of p38 MAPK phosphorylation by α-adducin gene silencing were not detectable (N = 4). In Western blot studies demonstrated that PV-induced α-adducin phosphorylation at Ser-726 was not blocked by specific inhibition of PKA using H-89 preincubation for 2 h (N = 3).

PKC does not participate in PV-IgG-dependent adducin phosphorylation because inhibition of PKA by preincubation with H-89 did not reduce the amount of phosphorylated adducin compared with PV-IgG treatment alone (Fig. 8D). Thus, we focused on PKC, which was shown to be rapidly activated following PV-autoantibody binding (34, 35) and is well known to phosphorylate adducin at Ser-726 (10). We applied the PKC inhibitor BIM-X in HaCaT cells, which pre-
vented adducin phosphorylation by the PKC activator PMA as well as phosphorylation of extracellular signal-regulated kinases-1/2 (ERK1/2), another well established PKC target (36) (Fig. 9A). PV-IgG-mediated phosphorylation of adducin after 10 min was abolished by simultaneous PKC inhibition via BIM-X (Fig. 9B). Furthermore, simultaneous incubation with BIM-X prevented cell dissociation induced by 24 h PV-IgG incubation (10.4 ± 1.3 versus 49.6 ± 3.1 fragments; Fig. 9C).

Conventional PKC isoforms require Ca²⁺ for activation (37) and it has previously been shown that PV-IgG induce a rapid increase in cytosolic Ca²⁺ levels (38). Similar in our study, a PV-IgG-specific increase in cytosolic Ca²⁺ was detectable by aequorin-based calcium assays (28) starting immediately after injection of autoantibodies and lasting ~180 s (Fig. 9D). Most importantly, preincubation with the cell-permeable Ca²⁺-chelating agent BAPTA-AM prevented both adducin and ERK

**FIGURE 9.** Adducin phosphorylation in response to PV-IgG is mediated via Ca²⁺-dependent PKC signaling. A, in immunoblot analysis, phosphorylation of adducin by incubation of the specific PKC activator PMA for 10 min was blocked by co-incubation of PKC inhibitor BIM-X. The effectiveness of PKC modulators was tested by ERK phosphorylation analyses (N = 4). B, PV-IgG-induced adducin phosphorylation after 10 min was abolished by co-treatment with BIM-X. C, similarly, simultaneous incubation with BIM-X prevented cell dissociation induced by 24 h PV-IgG incubation (N = 5, n > 10; *, p < 0.05 versus control; #, p < 0.05 versus PV-IgG). D, in aequorin-based Ca²⁺-measuring assays, a PV-IgG-specific rapid increase of cytosolic Ca²⁺ levels was detectable. E, Western blot analysis revealed that BAPTA-AM incubation for 4 h blocked both the PV-IgG-induced adducin phosphorylation as well as ERK-phosphorylation (N = 3). F, BAPTA-AM co-incubation for 4 h prevented loss of cell adhesion caused by PV-IgG (N = 4, n > 8; *, p < 0.05 versus control; #, p < 0.05 PV-IgG).
phosphorylation, the latter indicative of PKC activation (Fig. 9E). Similar to PKC inhibition, BAPTA-AM co-treatment pre-
vented cell dissociation induced by 4 h of PV-IgG incubation (18.0 ± 1.7 versus 31.8 ± 1.5 fragments; Fig. 9F). Collectively,
our data indicate that adducin phosphorylation in response to pemphigus autoantibodies is mediated via Ca\textsuperscript{2+}-dependent
PKC signaling.

**DISCUSSION**

**Adducin Regulates Keratinocyte Cell-Cell Cohesion**—Although regulation of the membrane cytoskeleton by adducin is well established (3, 4) and previous studies demonstrated association of adducin with intercellular contacts (7, 8), there is little insight into the functional role of adducin for intercellular adhesion. Here we show that adducin is indispensable for keratinocyte cohesion, which is in line with a study suggesting that depletion of α-adducin impairs E-cadherin-mediated adhesion of MDCK cells (39). In our study we demonstrate that specific α- or γ-adducin knockdown resulted in loss of keratinocyte cohesion and in reduction of Dsg3 protein content in the cyto-
skeleton-bound pool, interestingly without affecting E-cadherin. These data suggest that adducin controls keratinocyte cohesion specifically by regulating desmosomal Dsg3 levels. Previously, a direct interaction of Dsg3 and actin was demonstrated, however, in the non-cytoskeletal pool only and not in Dsg3 connected to the cytoskeleton (24). Nevertheless, a direct regulation of Dsg3 by adducin, *i.e.* as a scaffolding protein, is unlikely because we did not observe an interaction of adducin and Dsg3 in immunoprecipitation experiments (data not shown). Thus, it is likely that adducin modulates Dsg3 turnover via its influence on the cortical actin cytoskeleton (20).

An indirect regulation of Dsg3 levels by adducin is conceivable by modulating its turnover in desmosomes. Indeed, it has been shown that desmoplakin is transported to the membrane via an actin-driven mechanism on establishment of cell-cell contact (40), a process that may require plakophilin-2 and RhoA (41). Our data from FRAP experiments indicate that primarily the assembly pathway of Dsg3 to the desmosome is mod-
ulated by adducin. In this context, adducin may be involved in regulating the lateral incorporation of Dsg3 into desmosomes. Newly synthesized Dsg3 was shown to first be transported to the membrane and subsequently becoming incorporated into already existing desmosomes (42). The cortical actin cytoskel-
eton restricts lateral mobility and shapes clusters of membrane proteins by forming a net-like structure, with the actin fila-
ments building the fences (43–45). Adducin may serve to control this “corralling” function of the actin membrane cyto-
skeleton and thereby regulate lateral shifts of Dsg3 into desmosomes. It has been shown that PKC-mediated adducin phos-
phorylation at Ser-726 reduced actin binding and induced its redistribution away from the cell membrane (6). In line with this, we observed fragmentation of adducin staining at sites where PV-IgG caused intercellular gap formation. Thus, redistribution of adducin at sites where cell junctions are disrupted or reduced binding of adducin to actin may alter cortical actin dynamics that are required to promote Dsg3 incorporation into desmosomes. Up to now it is unclear why adducin affects Dsg3 levels but not the levels of E-cadherin. It is possible that the mechanisms by which desmosomal cadherins are targeted to the cell membrane are different compared with E-cadherin, or that the sorting of the adhesion molecules into their respective
Adducin and Keratinocyte Cohesion

junctioinal compartment requires distinct contributions of the actin cytoskeleton (46). The latter is supported by the recent finding that the assembly of desmosomes but not of adherens junctions is lipid raft-dependent (47). Further work is not only required to elucidate these aspects but also needs to investigate whether adducin regulation is restricted to Dsg3 or affects other desmosomal adhesion molecules as well.

Adducin Phosphorylation by PKC May Represent a Protective Mechanism in Response to Pemphigus Autoantibodies Which Is Augmented by RhoA—One unexpected observation was that both pemphigus autoantibodies (destabilizing cell adhesion) and RhoA activation (stabilizing cell adhesion) induced phosphorylation of adducin. We interpret these results that binding of pemphigus autoantibodies induces protective signaling within keratinocytes (Fig. 10, green arrows). This is underscored by the fact that the effect of AK23 was pronounced after introduction of the phosphorylation-deficient mutant of α-adducin. The concept of rescue mechanisms triggered by autoantibody binding is conceivable because it was previously shown that keratinocytes increase the amount of cAMP to reduce cell dissociation (48). Our data demonstrate that phosphorylation of adducin at Ser-726 is mediated, either directly or indirectly, by PKC. Furthermore, PKC activation in response to pemphigus autoantibodies is likely Ca²⁺-dependent. This is in line with previous studies implicating Ca²⁺/PKC signaling in pemphigus (38, 49, 50). Based on the fact that conventional PKC isoforms require Ca²⁺, it may be concluded that adducin phosphorylation in this context relies on these classical PKC isoforms. Our data demonstrate that PKC activation in response to autoantibody treatment results both in adhesion-protective signaling such as adducin phosphorylation as well as in cell dissociation (48). This is shown by our experiments in which both PKC inhibition as well as inhibition of Ca²⁺-signaling prevented cell dissociation after pemphigus autoantibody binding. Thus, the effect of PKC activation in response to pemphigus autoantibodies is complex (20, 51) (Fig. 10). Indeed, the dual function of PKC to promote and destabilize desmosomal adhesion is well recognized in the literature (52). For destabilization, PKC appears to render Dsg3 susceptible for autoantibody-induced depletion (34, 53) and prevent hyperadhesion (54), possibly by phosphorylation of desmoplakin (55, 56). For stabilization, plakophilin-2 was shown to recruit PKC to promote desmosome formation and keratin filament anchorage (57). At present it is unclear how the balance of stabilizing and destabilizing mechanisms in response to autoantibody binding is tuned.

RhoA probably is not involved in the phosphorylation of adducin in response to autoantibody treatment because RhoA activity was reduced at time points when adducin was phosphorylated. Nevertheless, pharmacological activation of RhoA by CNFy may enhance this protective pathway of adducin phosphorylation (Fig. 10). The present study extends our previous observations on the protective effect of RhoA-mediated actin modulation in pemphigus and identifies phosphorylation of adducin at Ser-726, possibly via Rho-kinase, to be important for strengthening cell adhesion. The fact that the protective effect of RhoA activation was only partially inhibited under conditions of adducin knockdown may be explained by the remaining adducin that was not targeted by siRNA. In addition, our previous findings demonstrated that activation of RhoA also prevents keratin retraction, a hallmark of pemphigus (16). In this study, p38 MAPK reduced RhoA signaling following PV-IgG incubation, which also would be expected to result in reduced adducin phosphorylation. This may, however, be masked by simultaneous PKC activation.

It is unclear whether PKC and Rho-kinase both directly phosphorylate adducin at Ser-726. This residue was initially shown to be phosphorylated by PKC and PKA (1, 10). Thus, it is possible that Rho-kinase acts upstream of PKC. The precise mechanisms of Ser-726 phosphorylation need to be determined in future studies.

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Adducin and Keratinocyte Cohesion

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