Desmosome Signaling

INHIBITION OF p38MAPK PREVENTS PEMPHIGUS VULGARIS IgG-INDUCED CYTOSKELETON REORGANIZATION*

Received for publication, February 4, 2005, and in revised form, April 13, 2005
Published, JBC Papers in Press, April 19, 2005, DOI 10.1074/jbc.M501365200

Paula Berkowitz‡, Peiqi Hu‡, Zhi Liu‡, Luis A. Diaz‡, Jan J. Enghild§, Michael P. Chuang§, and David S. Rubenstein‡‡**

From the Departments of §Dermatology and ¶Cell and Molecular Physiology and the ||Lineberger Comprehensive Cancer Center University of North Carolina-Chapel Hill School of Medicine, Chapel Hill, North Carolina 27599 and the ¶Department of Molecular Biology, University of Aarhus, DK-8000 Århus, Denmark

In the human autoimmune blistering disease pemphigus vulgaris (PV) pathogenic antibodies bind the desmosomal cadherin desmoglein-3 (dsg3), causing epidermal cell-cell detachment (acantholysis). Pathogenic PV dsg3 autoantibodies were used to initiate desmosome signaling in human keratinocyte cell cultures. Heat shock protein 27 (HSP27) and p38MAPK were identified as proteins rapidly phosphorylated in response to PV IgG. Inhibition of p38MAPK activity prevented PV IgG-induced HSP27 phosphorylation, keratin filament retraction, and actin reorganization. These observations suggest that PV IgG binding to dsg3 activates desmosomal signal transduction cascades leading to (i) p38MAPK and HSP27 phosphorylation and (ii) cytoskeletal reorganization, supporting a mechanistic role for signaling in PV IgG-induced acantholysis. Targeting desmosome signaling via inhibition of p38MAPK and HSP27 phosphorylation may provide novel treatments for PV and other desmosome-associated blistering diseases.

Changes in cell adhesion structures activate cellular signaling systems that link alterations in the state of cell adhesion to changes in cell behavior. Signaling downstream of adherens junctions (1–4) and integrins regulates differentiation and proliferation when cell-cell or cell-substrate adhesion is altered. Like other cell adhesion structures, the desmosome is a macromolecular complex whose components include cadherin, Armadillo, and plakin family proteins (reviewed in Ref. 5). Desmosome-mediated cell-cell adhesion is disrupted in the autoimmune blistering disease pemphigus vulgaris (PV),† characterized clinically by suprabasilar blisters of skin and oral mucosa. Anti-epidermal autoantibodies, present in PV patient sera and easily detected by immunofluorescence (IF) on peripheral sera and pathogenic PV IgG reproduces the clinical, histologic, and immunologic features of the disease when passively transferred to neonatal mice. Pathogenic PV autoantibodies bind the dsg3 ectodomain in a calcium-dependent immunological reaction (6).

Proposed mechanisms for PV IgG-induced keratinocyte detachment include (a) proteinase activation, (b) steric hindrance, and (c) activation of transmembrane signaling that down-regulates cell-cell adhesion (7–11). Previous work has suggested that PV IgG may activate intracellular signaling events; however, the precise nature and biological consequences of these events and their relationship to the mechanism of PV IgG-induced acantholysis remain unknown (7, 8). The specificity of anti-dsg3 antibodies in PV patient sera enabled us to utilize this reagent to initiate changes in desmosome structure and look for activation of signaling and the relationship of these signaling events to the mechanism of acantholysis.

EXPERIMENTAL PROCEDURES

Materials—HSP27 antibodies were from ABR (Golden, CO); phospho-HSP27, p38MAPK, and phospho-p38MAPK antibodies were from Cell Signaling Technology (Beverly, MA). SB202190, SB203580, Calphostin C, H-7, and U-73122 were from Calbiochem.

IgG Preparation—PV sera (PV1, mucocutaneous PV; PV2, mucosal PV) were previously described (12). PV IgG was purified from sera by ammonium sulfate precipitation followed by affinity chromatography on Protein G (HiTrap; Amersham Biosciences). IgG fractions were diazotized against PBS, sterile filtered, and CaCl2 was added to a final concentration of 0.5 mM. Purity was confirmed by SDS-PAGE and activity assayed by indirect IF on sectioned monkey esophagus (PV1 = 1.640, PV2 = 1.5120). Control IgG (no activity by indirect IF) was prepared in parallel from normal human sera. Depleted PV IgG was prepared by removal of dsg3-specific antibodies from sera PV2 by affinity chromatography on a recombinant dsg3 ectodomain column (12). The flow-through was free of dsg3-specific IgG by negative indirect IF (data not shown).

Tissue Culture—Normal primary human keratinocytes were passaged and expanded as described (13). Third passage keratinocytes were grown to 80–90% confluence when dsg3, but not dsg1, was detected by Western blot of cell extracts using dsg3- and dsg1-specific monoclonal antibodies, respectively (data not shown). [32P]H3PO4 was then added to the culture medium (5 M Ci/ml); the cells were incubated for 2 h and either control IgG, PV IgG, or an equivalent volume of buffer was added (to concentrations of 5, 10, or 25 μM). Cells were incubated for 15, 30, or 60 min and then washed extensively in PBS prior to harvesting in IEF lysis buffer (8 M urea, 4% CHAPS, 2.5 mM dithiothreitol, 40 mM Tris, 10 mM HEPES, 100 mM leupeptin, 10 μM E-64, 1 mM phenylmethylsulfonyl fluoride). Protein concentration was by modified Bradford as described (13). IPG buffer (pH 3–10, non linear; Amersham Biosciences) was added to each sample to a final concentration of 0.5% prior to isoelectric focusing. For inhibition studies, keratinocytes were preincubated for 60 min at 37 °C with the p38MAPK inhibitors SB202190 (100 μM) or SB203580 (100 μM), the protein kinase C inhibitors Calphostin C (100 nM) or H-7 (10 μM), or the phospholipase C inhibitors.

This paper is available on line at http://www.jbc.org

23778

This is an Open Access article under the CC BY license.
inhibitor U-73122 (4 μM), after which PV IgG was added to a final concentration of 25 μM and the cells were incubated for 30 min at 37 °C and harvested in IEF lysis buffer.

Two-dimensional Gel Electrophoresis—40 μg of 32P-labeled extracts per gel were separated in the first dimension using 13 cm pH 3–10, non-linear IPGphor strips (Amersham Biosciences) and in the second dimension by 10% SDS-PAGE. Gels were dried and exposed to x-ray film and phosphorimage detection on an Amersham Biosciences Storm 840 phosphorimager. The ImageQuant (Amersham Biosciences) program was used to quantify the radioactive signal detected from each spot on individual gels. The signal detected by the phosphorimager is expressed as spot volume and corresponds to the radioactive decay from the 32P-labeled protein in each spot. Statistical significance (p < 0.05) was determined using the Student’s t test. Standards of known specific activity were spotted onto filter paper and used as internal controls. Spots whose radioactivity remained constant among the various conditions served as internal controls.

Protein Identification—Two-dimensional gel spots of interest were excised and digested with sequence grade trypsin and MALDI-TOF mass spectrometry data acquired using a Q-tof Ultima Global instrument (Micromass/Waters Corp., Manchester, UK) as previously described (14).

Native Gel Electrophoresis and Chemical Cross-linking—Keratinocytes grown to 80% confluence were incubated in the presence of 25 μM control IgG, 25 μM PV IgG, or an equivalent volume of buffer for 30 min and washed and harvested in PBS, and native extracts were prepared by Dounce homogenization. Extracts (10 μg) from each sample were separated on two-dimensional gels, and radioactivity in each spot was quantified by phosphorimage analysis. Each data point represents the average of three independent experiments; S.D. is shown.

FIG. 1. PV IgG-induced protein phosphorylation is dose dependent.
Normal human keratinocytes, cultured in the presence of [32P]H3PO4, were exposed to PV IgG, IgG, or buffer for 30 min at 37 °C, and extracts were separated by two-dimensional gel electrophoresis. A, representative autoradiograms (25 μM IgG). Relative to buffer and normal IgG controls, the PV IgG-treated keratinocytes show increased phosphorylation in the proteins corresponding to Spots 2, 3, and 5. In contrast, no notable difference in Spots 1 and 4 was observed between PV IgG- and control-treated cells. Phosphorylation of spots labeled with an asterisk did not change across time, dose, or culture conditions and serve as internal controls. B, enlargement of two-dimensional gel regions of interest. C, dose dependence. Keratinocytes, cultured in [32P]H3PO4, were exposed to 5, 10, or 25 μM PV IgG (shaded bars) or control IgG (clear bars) for 30 min at 37 °C. Extracts were separated on two-dimensional gels, and radioactivity in each spot was quantified by phosphorimage analysis. Each data point represents the average of three independent experiments; S.D. is shown.

Desmosome Signaling 23779
trotransferred to polyvinylidene difluoride, and subjected to Western blot analysis using anti-HSP27 antibodies.

Confocal Microscopy—Keratinocytes grown to 100% confluence were incubated with 25 μM PV IgG (PV IgG) for 6 h at 37 °C or preincubated for 60 min at 37 °C with the p38MAPK inhibitor SB202190 (100 μM) and then incubated with 25 μM control IgG (Con IgG) or 25 μM PV IgG (PV IgG) for 6 h at 37 °C. Cells were fixed and stained with fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR), pancytokeratin antibodies (clone AE1/AE3; Zymed Laboratories Inc., San Francisco, CA), or goat anti-human E-cadherin antibodies (R & D Systems, Inc., Minneapolis, MN) as previously described (16), followed by Cy2- and Cy3-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA). Images were analyzed using a Leica SP2 AOBS confocal microscope with an excitation wavelength of 488 nm and capture at 500−550 nm or an excitation wavelength of 561 nm and capture at 590−650 nm using a ×63 objective with NA 1.4. Double-labeled samples were checked for bleed-through by turning off the longer wavelength laser and assaying for the absence of image. For three-dimensional image reconstructions, serial sections were scanned at 0.244 μm and rendered using the program Volocity Version 3 (Improvision, Lexington, MA).

RESULTS

We used dsg3-specific PV IgG to initiate structural changes in the desmosome and examined keratinocyte extracts for changes in intracellular phosphorylation by culturing normal human keratinocytes in the presence of [32P]H3PO4 and exposing them to PV IgG. Phosphorimage analysis of two-dimensional gel electrophoresis of [32P]-labeled keratinocyte extracts was used to identify and quantify changes in protein phosphorylation.

PV IgG Alters Cellular Phosphorylation Patterns—At least three phosphorylation events, which precede loss of cell-cell adhesion as assessed by membrane retraction of apposing cells, occurred within 30 min of exposure of keratinocytes to PV IgG (Fig. 1). Relative to buffer and normal IgG controls, PV IgG-treated keratinocytes show increased phosphorylation in the proteins corresponding to Spots 2, 3, and 5 (Fig. 1). No significant difference in phosphorylation was observed in Spots 1 and 4 in control or PV IgG-treated keratinocytes. Additionally, the phosphorylation of several spots (labeled with an asterisk in Fig. 1) did not change across time, dose, or culture conditions, and they serve as internal controls.

PV IgG-induced Protein Phosphorylation Is Dose Dependent—To examine dose dependence, keratinocytes were incubated in the presence of 5, 10, or 25 μM PV or control IgG or buffer and examined for changes in phosphorylation by two-
The autoradiograms of the 32P-labeled extracts (Fig. 1). Non-phosphorylated isoform (P0), anti-HSP27 (keratin) and anti-pan keratin (IgG-treated (Con IgG), mock-treated (PBS Con), or control incubated with PV IgG show increased phospho-HSP27 immunoreactivity. In-gel tryptic digestion and MALDI-TOF were used to determine the identity of Spot 2 as HSP27 (data not shown). We then determined the time course by culturing keratinocytes in the presence of [32P]H3PO4 and 25 μM p38MAPK results in phosphorylation of MAP kinase-activated protein kinase-2 (p38MAPK) inhibition of p38MAPK inhibited the phosphorylation of HSP27 in keratinocytes exposed to PV IgG (Fig. 4B). No notable inhibition of HSP27 phosphorylation was observed in the presence of the protein kinase C inhibitors Calphostin C and H-7 or the phospholipase C inhibitor U-73122. PV IgG-induced phosphorylation of HSP27 is associated with transitions from large to small oligomers (15, 17), we investigated the effects of PV IgG-induced HSP27 phosphorylation on its oligomerization. On native pore limit gels, both large and small oligomers of HSP27 were detected in human keratinocytes incubated with buffer or control IgG; however, a decreased level of large oligomeric HSP27 was observed in PV IgG-treated keratinocytes (Fig. 4C). The migration on native gels is consistent with prior reports of large HSP27 oligomers of average molecular mass 530 kDa reduced to small oligomers of molecular mass 110 kDa by phosphorylation with MAPKAP kinase 2 (17).

The large oligomeric isoform was not well detected by Western blot of native gels, perhaps because of the relative inefficiency of high molecular mass native proteins to undergo electrophoresis; therefore, cross-linking was used to stabilize large oligomeric HSP27. Native extracts from keratinocytes were chemically cross-linked in glutaraldehyde and subjected to reducing SDS-PAGE and immunoblot analysis with HSP27 antibodies. This protocol enhanced the detection of high molecular mass HSP27 oligomers observed in buffer or control IgG-treated cells but markedly reduced in PV IgG-treated keratinocytes (Fig. 4D).

p38MAPK Inhibition Blocks PV IgG-induced Keratin and Actin Reorganization—In response to PV IgG, the keratin filament network of keratinocytes retracts from the cell membrane. We utilized this physiologic end point to determine whether the observed signaling events had a role in activating PV IgG-induced changes in the cytoskeleton. Consistent with previous reports (11), keratin filaments in PV IgG-treated keratinocytes were retracted from the membrane and demonstrated enhanced perinuclear localization. PV IgG-induced keratin filament retraction was prevented by the p38MAPK inhibitor SB202190 (Fig. 5A). Furthermore, in PV IgG-treated keratinocytes phalloidin staining suggested that the actin cytoskeleton was undergoing reorganization consistent with the transition from stationary adherent cells to non-adherent migratory cells. PV IgG treatment was associated with a change from cortical staining to a pattern suggestive of ruffling membranes (18) (Fig. 5B). Notably, the PV IgG-induced actin reorganization was similarly prevented by the p38MAPK inhibitor SB202190 (Fig. 5B, PV IgG + Inh).

**Discussion**

Although the acantholytic properties of dsg3 autoantibodies from PV patient sera have been well described, the molecular mechanisms by which these autoantibodies disrupt keratinocyte cell-cell adhesion has not been characterized. By incubating keratinocytes in the presence of [32P]H3PO4, we were able to identify changes in the phosphorylation pattern of cellular substrates after addition of purified PV IgG to keratinocyte cell cultures.

Several spots, resolved by two-dimensional gel electrophoresis, were observed to undergo rapid changes in phosphorylation. The increase and subsequent decrease in phosphorylation observed is consistent with a dynamic regulatory process and with the transient nature of protein post-translational modifications characteristic of intracellular signaling cascades. Other spots on the gel were not affected by the addition of PV IgG; i.e. no time- or dose-dependent phosphorylation changes were ob-
p38MAPK is phosphorylated in PV IgG-treated keratinocytes. Cells were exposed to 25 μM PV IgG, control IgG, or buffer for 30 min at 37 °C. Extracts (15 μg protein/lane) were separated by 10% SDS-PAGE, followed by immunoblotting with antibodies to HSP27, phospho-HSP27, p38MAPK, and phospho-p38MAPK. Increased phospho-HSP27 and phospho-p38MAPK immunoreactivity is detected in PV IgG-treated cells. B, inhibitors of p38MAPK block PV IgG phosphorylation. Cells were preincubated for 60 min at 37 °C with p38MAPK inhibitors SB202190 or SB203580, protein kinase C inhibitors Calphostin C or H-7, or phospholipase C inhibitor U-73122, after which PV IgG (25 μM) was added, and the cells were incubated for 30 min at 37 °C and harvested in IEF lysis buffer. Extracts were separated on 10% SDS-PAGE, followed by immunoblotting with anti-HSP27 and anti-phosphoHSP27 antibodies.

By native pore limit gel electrophoresis, both large and small oligomers of HSP27 were detected in extracts (10 μg/lane) of cells incubated with buffer or control IgG; however, decreased levels of large oligomeric HSP27 were observed in PV IgG cells. D, large oligomeric HSP27 is readily visualized in buffer and control IgG-treated, but not PV IgG-treated, keratinocytes. Native extracts chemically cross-linked and subjected to reducing SDS-PAGE (5 μg protein/lane) and immunoblot analysis with HSP27 antibodies enhanced the detection of high molecular weight HSP27 oligomers whose levels were reduced in PV IgG-treated cells.

The observation that HSP27 is one of the proteins phosphorylated in response to PV IgG is intriguing. HSP27 functions as a molecular chaperone to facilitate the refolding of denatured proteins but also participates in signaling where it may regulate elements of the cytoskeleton, including actin filaments (19–21) and keratin intermediate filaments (22). Large oligomeric HSP27 is thought to have chaperone function, whereas, small oligomeric HSP27 may have direct signaling and cytoskeletal regulatory functions (22, 23). Phosphorylation of HSP27 is thought to have a role in its ability to regulate the cytoskeleton (24–26). Missense mutations in HSP27 lead to disrupted neurofilament assembly and cause the neuromuscular disorder Charcot-Marie Tooth disease as well as distal hereditary motor neuropathy, providing additional support for the role of HSP27 in intermediate filament regulation (27).

Our observations are consistent with a mechanism by which PV IgG binding to desmoglein 3 activates “outside-in” desmosomal signaling in which (i) phosphorylation of p38MAPK leads to (ii) phosphorylation of MAPKAP kinase 2, which in turn (iii) phosphorylates HSP27, leading to (iv) changes in HSP27 quaternary structure and cytoskeletal rearrangements (Fig. 5C). Interestingly, endorepellin binding to α6β1 integrin activates a signaling pathway in which p38MAPK and HSP27 phosphorylation are associated with structural changes in the actin cytoskeleton (28), suggesting that HSP27 may have a central role in cell adhesion junction signaling. The observation that specific inhibition of this pathway using p38MAPK inhibitors prevents changes in the cytoskeleton associated with the transition from an adherent to non-adherent phenotype supports the hypothesis that the observed signaling events may be required for the loss of cell-cell adhesion induced in vivo by PV autoantibodies.
HSP27 phosphorylation correlates with changes induced by PV IgG; however, it may be premature to suggest that HSP27 phosphorylation is the critical step in this signaling pathway. Other p38MAPK substrates may play a role in PV IgG-induced cytoskeletal reorganization. Numerous direct and indirect downstream targets of p38MAPK have been reported, including protein kinases (29–31), transcription factors (32–34), and cytoskeletal proteins (35, 36). Furthermore, although we have not yet determined the protein identity of Spots 3 and 5, it seems likely that they also play a role in this response because they too exhibit dose- and time-dependent phosphorylation upon addition of PV IgG.

Keratinocytes from plakoglobin knock-out mice form desmosome-like structures that appear to mediate cell-cell adhesion in monolayer cultures but are resistant to PV IgG-mediated keratin filament retraction (11). This observation suggests that mechanisms in addition to steric hindrance may be required for PV IgG to mediate the loss of keratinocyte cell-cell adhesion and provides additional support for the hypothesis that signaling plays a role. Further support for desmosome signaling is provided by the observation that genetic defects in desmosome components are associated with altered epithelial differentiation (37, 38).

Binding of specific pathogenic antibodies to desmoglein 3 activates intracellular phosphorylation events, suggesting that, in addition to functioning in cell-cell adhesion, desmosomes are capable of acting as transmembrane receptors that transduce signals from the extracellular environment to the intracellular environment. Pharmacologic inhibition of PV IgG-induced p38MAPK activation and HSP27 phosphorylation may prove beneficial in the treatment of this severe and life-threatening autoimmune disease.

Acknowledgments—We thank Nikki Duncan and Drs. Lowell Goldsmith, Ning Li, Mark Peifer, and Simon Warren.

REFERENCES

1. Hu, P., Berkowitz, P., O'Reeke, E. J., and Rubenstein, D. S. (2003) J. Invest. Dermatol. 121, 242–251
