Generation and Characterization of Monoclonal Antibodies Against the Proregion of Human Desmoglein-2

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Abstract

Cadherins are synthesized with a signal sequence and a proregion that must be removed for optimal adhesive activity. Mutations that prevent processing of cadherins have been implicated in a number of human diseases; thus understanding their processing is critical. In this study, we produced and characterized a number of monoclonal antibodies against the proregion of the desmosomal cadherin, human desmoglein-2, that will facilitate investigations into the processing of this protein.

Introduction

DESMOSOMES ARE INTERCELLULAR JUNCTIONS found in tissues like skin and heart that are subjected to significant mechanical stress. Desmosomes, which provide strong cell-cell adhesion by linking to the intermediate filament cytoskeleton, are comprised of proteins from three families: the cadherin family, the armadillo family, and the plakin family.^(1,2) Desmosomal dysfunction has been implicated in a number of diseases, including striate palmoplantar keratoderma, skin fragility, and ectodermal dysplasia,⁽³⁾ and most recently arrhythmic right ventricular cardiomyopathy (ARVC).⁽⁴⁾

The desmosomal cadherins, desmogleins and desmocollins, mediate calcium-dependent cell-cell adhesion by forming homotypic and heterotypic interactions with one another.^(5,6) Desmosomal cadherins are synthesized with a signal peptide that directs them to the endoplasmic reticulum and a proregion that is removed during protein processing. The mature protein includes four highly conserved extracellular domains (EC 1–4) and a fifth membrane proximal, more variable EC domain that is referred to as the “extracellular anchor domain.”⁽⁷⁾ A cell adhesion recognition motif in EC1 contributes to the adhesive function of the desmosomal cadherins.⁽⁸⁾

Very little is known about the proteolytic processing of desmosomal cadherins, or how important processing is to the generation of a functional adhesive protein. However, we can infer the importance of processing from studies done on classic cadherins, which are also synthesized as immature proteins with a signal sequence and a proregion, which must be removed to generate a mature functional cad-

herin.^(9–11) Studies on the processing and transport of classic cadherins to the plasma membrane have been facilitated by antibodies that specifically recognize immature forms of these cadherins, without cross-reactivity to the mature forms. A lack of reagents that specifically recognize immature forms of the desmosomal cadherins has hampered studies on the importance of their processing. Interestingly, mutations that likely prevent proteolytic removal of the proregion of desmoglein-2 have been identified in patients with ARVC, suggesting that impaired desmosomal cadherin function caused by defective processing contributes to the cardiac defects seen in these patients.⁽⁴⁾ All of the classic and desmosomal cadherins are predicted to have N-terminal pro sequences immediately C-terminal of their signal sequence. The pro sequences vary in length, but all of them terminate in consensus sites for proprotein convertases immediately upstream of EC1. Thus, to better understand the processing and trafficking of desmosomal cadherins, we developed monoclonal antibodies against the proregion and EC1 of desmoglein-2.

Materials and Methods

Reagents, antibodies, and cells

All reagents were from Sigma (St. Louis, MO) unless otherwise noted. The mouse monoclonal antibody against desmoglein-2 extracellular domain (6D8) has been described⁽¹²⁾; 6D8 recognizes EC4 of human desmoglein-2 (unpublished data, My Mahoney, Thomas Jefferson University, Philadelphia, PA). The mouse monoclonal antibody against the cytoplasmic domain of desmoglein-2 (DG3.1) was developed by Werner Franke's laboratory (German Cancer Re-

University of Nebraska Medical Center, Departments of ¹Genetics, Cell Biology and Anatomy; ²Oral Biology; ³Eppley Institute for Research in Cancer and Allied Diseases; ⁴Eppley Cancer Center, Omaha, Nebraska.

search Center, Heidelberg, Germany) and purchased from Research Diagnostics (Flanders, NJ). Monoclonal anti-tubulin (E7) was from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). A431 human epidermal carcinoma cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT).

Detergent extraction of cells

Confluent monolayers of cells were washed with phosphate-buffered saline (PBS) three times, placed on ice, scraped, and triturated vigorously for 1 min in Empigen buffer (10 mM Tris-acetate [pH 8.0], 0.1% Empigen BB [Calbiochem, San Diego, CA], 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride [PMSF]) or TNE buffer (10 mM Tris-acetate [pH 8.0], 0.5% NP-40, 1 mM EDTA, 2 mM PMSF). Insoluble material was pelleted by centrifugation at 14,000 *g* for 15 min at 4°C, and the supernatant stored at -80°C. For analysis of TNE insoluble material, the pellet was washed one time with TNE buffer and resuspended in Laemmli sample buffer.⁽¹³⁾

Immunoblots

Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred overnight onto nitrocellulose membranes and blocked in TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween-20) supplemented with 5% non-fat dry milk or 4% bovine serum albumin (BSA). Membranes were washed three times for 15 min in TBST and incubated in primary antibody for 1 h; washed three washes in TBST for 15 min; and incubated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse IgG diluted to 1:10,000 (Jackson ImmunoResearch West Grove, PA). Excess antibody was removed by washing three times with TBST for 15 min. For AP-conjugated antibodies, the immune complexes were identified using Nitro BT and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt. For HRP-conjugated secondary antibodies, the immune complexes were identified using Pierce Supersignal Substrate (Pierce Chemical, Rockford, IL).

Immunofluorescence microscopy

Cells grown on glass coverslips (75% confluent) were rinsed briefly in PBS, fixed using Histochoice Tissue Fixative (Amresco, Solon, OH) for 30 min, rinsed briefly in PBS, and blocked in PBS containing 10% heat-inactivated goat serum for 15 min. Coverslips were incubated in primary antibody for 1 h at room temperature, followed by 1 wash in blocking solution and 3 washes in PBS. Coverslips were incubated in fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch), diluted in blocking solution for 1 h at room temperature followed by one wash in blocking solution and three washes in PBS, and mounted in Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were collected on a Zeiss Axiovert 200 M (Carl Zeiss MicroImaging, Thornwood, NY) equipped with an ORCA-ER (Hamamatsu, Houston, TX) digital camera and processed using SlideBook software (Intelligent Imaging Innovations, Santa

Monica, CA). For cell surface immunofluorescence microscopy, cells were grown on glass coverslips, and living cells were incubated on ice with primary antibodies. Unbound antibody was washed away, and the cells were processed as above.

Generation of desmoglein-2 monoclonal antibodies

PCR was used to generate the antigen (amino acids 37-164 GenBank accession NM_001943), which included a portion of the proregion and the entire extracellular domain 1 (EC1) of desmoglein-2 fused to GST. The primers were 5'-GAC-CATGGCACTGCTTCCTAAACATCCTC-3' and 5'-GTCTC-GAGTTATGCAAAGACATCCTGTGTG-3'. The PCR product was cloned into pCR2.1TOPO and sequenced to ensure that no errors occurred, and the NcoI-XhoI fragment was ligated into pGST-parallel 1.⁽¹⁴⁾ The desmoglein-2 fusion protein was expressed in *E. coli* and purified by affinity chromatography. Three 8- to 9-week-old female Balb/C mice were injected subcutaneously with 50-150 µg antigen per mouse. Intraperitoneal booster injections of 50-150 µg were given at 2-week intervals, followed by daily injections 3 days prior to sacrifice. Splenocytes were isolated, incubated 10 min on ice in Sigma red cell lysis buffer to remove the red blood cells, and fused with the mouse myeloma cell line P3/NS1/1-Ag4-1 (ATCC) in the presence of polyethylene glycol (ATCC). The complete fusion was plated in 30 96-well plates, and medium containing hypoxanthine aminopterin and thymidine was added the following day to eliminate unfused myeloma cells. Hybridoma supernatants were screened by immunoblot and immunofluorescence. Positive hybridomas were cloned by limiting dilution and maintained in Sigma HY medium supplemented with 20% FBS. Monoclonal antibodies were isotyped using an MMT1 kit from AbD Serotec (Oxford, United Kingdom). All six antibodies were IgG1 with κ light chain.

Epitope mapping

GST-tagged PCR-generated constructs were used to map the epitopes recognized by the monoclonal antibodies (Fig. 1). GST-pro is comprised of desmoglein-2 amino acids 24-50, which spans the entire proregion; GST-proEC1N is comprised of amino acids 24-112, which includes the proregion and an N-terminal portion of EC1; GST-proEC1 is comprised of amino acids 24-164, which includes the proregion and all of EC1; GST-EC1N is comprised of amino acids 50-112, which is the N-terminal portion of EC1. Primers for the proregion were 5'-GACCATGGCACTTCACTTACAGGTCTTA-3' and 5'-GTCTCGAGTTATGCGCGCTTTTGCCGCAC-3'; primers for the proregion + the N-terminal portion of EC1 were 5'-GAC-CATGGCACTTCACTTACAGGTCTTA-3' and 5'-GTCTC-GAGTTATGCATTAGTTCTCCAG-3'; primers for the proregion + all of EC1 were 5'-GACCATGGCACTTCACTTACAGGTCTTA-3' and 5'-GTCTCGAGTTATGCAAAGACATCCTGTGTG-3'; primers for the N-terminal portion of EC1 were 5'-GACCATGGCCTGGATCACCGCCCCC-3' and 5'-TCTCGAGTTATGCATTAGTTCTCCAG-3'. PCR products were cloned into pCR2.1TOPO, sequenced to verify that no errors were introduced, and ligated into pGST-parallel 1. The constructs were expressed in *E. coli* and crude lysates were used for mapping.

FIG. 1. Constructs used for epitope mapping. In addition to GST, the constructs included GST fused to amino acids 24–50 of desmoglein-2 (GST-pro); GST fused to amino acids 50–112 of desmoglein-2 (GST-EC1N); GST fused to amino acids 24–112 of desmoglein-2 (GST-proEC1N); and GST fused to amino acids 24–164 of desmoglein-2 (GST-proEC1).

shRNA knockdown of desmoglein-2

To generate a desmoglein-2 specific shRNA targeting nucleotides 190–208 of NM_001943, two oligos were synthesized by the Eppley Cancer Center Molecular Biology Core Facility (Omaha, NE): 5'-GATCCCCGAGAGGATCTGTC-CAAGAATTCAAGAGATTCTTGGACAGATC-CTCTCTTTT3' and 5'-AGCTTAAAAAGAGAGGATCTGTCCAAGAATCTCTTGAATTCTTGGACAGATCCTCTC GGG 3'. The oligos were mixed 1:1, heated to 95°C for 5 min, and allowed to cool slowly to room temperature. The annealed oligos were ligated into pSuper Retro-puro (Oligo-Engine, Seattle, WA), digested using BglII and HindIII, and sequenced to verify that the sequence was correct. Retrovirus production and infection was performed essentially as described previously.⁽¹⁵⁾ Briefly, Phoenix cells (5×10^5 /100 mm dish) were transfected using calcium phosphate (Stratagene, La Jolla, CA), and 48 h later were switched to medium containing 2 μ g/mL puromycin for selection. Puromycin-resistant cells were grown in DMEM supplemented with 10% FBS lacking puromycin at 32°C for 24 h for virus production. Viral-conditioned medium was collected and passed through a 0.45 μ m filter and polybrene was added to 4 μ g/mL. A431 cells (2×10^5 cells/well) were plated in 6-well dishes 18 h prior to infection. Fresh viral conditioned medium was added to target cells for 6 h at 32°C, the medium was replaced with fresh medium, and cells were returned to 37°C. Two days after infection, selective medium containing 2 μ g/mL puromycin was added and a population of retrovirally infected cells was isolated. Control (mock) A431 cells were infected with an empty virus.

Cell surface biotinylation

Cells were grown to confluence in 10 mm dishes, washed with PBS, and incubated with the non-membrane-permeable biotinylation reagent sulfo-N-hydroxysuccinimidobiotin (0.2 mg/mL) (Pierce Chemical) for 10 min on ice. After quench-

ing with 5 mL DMEM containing 10% FBS and washing with PBS, the cells were lysed directly on the dish with TNE buffer. The lysates were incubated with primary antibody for 2 h at 4°C with rotation. Anti-mouse IgG agarose beads were added to the extracts and the mixture incubated 1 h at 4°C with rotation. The beads were then washed three times with TBST, mixed with Laemmli sample buffer, and placed in boiling water for 5 min; the proteins were resolved by SDS-PAGE. The membrane was blocked with 4% BSA and probed with HRP-labeled streptavidin (Vectastain ABC kit, Vector Laboratories).

FIG. 2. Epitope mapping of anti-desmoglein monoclonal antibodies. Proteins encoded by the constructs shown in Figure 1 were resolved by SDS-PAGE and immunoblotted with unpurified supernatant from the various hybridomas. The antibodies fell into three classes. (A) Class I antibodies (3B1 and 20G1) recognize the proregion of desmoglein-2, as they immunoblot proteins that include the proregion, even GST-pro, which is comprised of only GST and the proregion. (B) Class II antibodies (7H9 and 19B9) recognize proteins that include EC1 linked to another amino acid, whether this is from the proregion or from GST. (C) Class III antibodies (10D2 and 13B11) are specific for EC1.

Immunoprecipitation

All polypropylene tubes were rinsed with 0.01% Triton X-100 and dried prior to use in immunoprecipitations. Cell extracts were prepared as described above. 300 μ L hybridoma

supernatant, or the manufacturer's recommendation for purchased antibodies, was incubated with 50 μ L packed anti-mouse IgG affinity gel for 1 h at 4°C. Excess antibody was removed by washing three times with TBST, and the beads were incubated with cell extract for 1 h at 4°C. Beads were

FIG. 3. Antibodies that recognize the proregion of desmoglein-2 a protein doublet. (A) A431 cell extract (50 μ g protein) was resolved by SDS-PAGE and immunoblotted with unpurified supernatant from the antidesmoglein-2 hybridomas. Class I and II antibodies recognize a doublet on immunoblots, while class III antibodies recognize a single band. (B) A431 cell extract (50 μ g protein) was resolved by SDS-PAGE, transferred to nitrocellulose, and strips were cut in the middle of lanes (indicated by black vertical bars) to better characterize the mobility of the bands recognized by the various antibodies. The single band recognized by class III antibodies migrates between the doublet bands recognized by class I and II antibodies. (C) A431 cell extract (250 μ g protein) was immunoprecipitated with class I or II antibodies (300 μ L unpurified hybridoma supernatant). The immunoprecipitated protein were resolved by SDS-PAGE and immunoblotted with DG3.1. (D) Extracts of A431 cells (50 μ g protein) mock infected or infected with virus encoding shRNA against desmoglein-2 were resolved by SDC-PAGE and immunoblotted with the various antidesmoglein monoclonal antibodies using unpurified hybridoma supernatant.

washed five times with TBST, mixed with 50 μ L Laemmli sample buffer, and placed in boiling water for 5 min, and proteins were then resolved by SDS-PAGE. To immunoprecipitate desmosomal cadherins from culture medium, cells were grown in T25 flasks for 2 days and culture medium was used in the immunoprecipitation reaction as described above.

Results

Generation of antibodies against the proregion/EC1 of desmoglein-2

Balb/C mice were immunized with a GST-tagged recombinant protein that included a portion of the proregion and EC1 of human desmoglein-2. Hybridomas were prepared as previously described.⁽¹⁶⁾ Six positive hybridomas that produced antibodies recognizing the recombinant antigen but not GST on immunoblots were chosen for further study.

To determine the epitopes recognized by the six antibodies, we used a panel of GST-tagged recombinant proteins: GST alone, GST fused to the proregion of desmoglein-2, GST fused to the N-terminal 63 amino acids of EC1 of desmoglein-2, GST fused to the proregion plus the N-terminal 63 amino acids of EC1 of desmoglein-2, and GST fused to the proregion plus the entire EC1 of desmoglein-2 (Fig. 1). Antibodies 3B11 and 20G1 recognized fusion proteins that included the entire proregion but not fusion proteins missing the proregion (Fig. 2A), indicating these antibodies specifically recognize epitopes in the proregion of desmoglein-2. These antibodies were designated class I antibodies. Antibodies 7H9 and 19B9 recognized fusion proteins that included the most N-terminal amino acids of EC1, whether or not they included the proregion (GST-EC1N, GST-proEC1N, and GST-proEC1), but not to a fusion protein comprised of GST and the proregion, but missing EC1 (GST-pro; Fig. 2B). For reasons described below, we have evidence that these antibodies recognize a determinant that is right at the fusion between EC1 and the proregion and thus are "proregion dependent" and were designated class II. Antibodies 10D2 and 13B11 recognized only the fusion protein that included the most C-terminal amino acids of EC1 (GST-proEC1; Fig. 2C), indicating they were specific for EC1 and completely in-

dependent of the proregion. These antibodies were designated class III.

Proregion-dependent antibodies recognize a doublet

A431 cells were lysed with Empigen BB to efficiently extract desmosomal proteins, and the extracts resolved by SDS-PAGE as described.⁽¹⁶⁾ The four proregion-dependent antibodies (3B11, 20G1, 7H9, and 19B9) immunoblotted a doublet (Fig. 3A). In contrast, the class III antibodies 10D2 and 13B11 that map to the C-terminal portion of EC1 recognized a single band that co-migrated with the band recognized by 6D8, which binds to EC4. When cell extracts were resolved for an extended period of time on a lower percent acrylamide gel, it was clear that class III antibodies recognize a band that migrates in between the doublet bands recognized by class I and II antibodies (Fig. 3B).

Epitope mapping clearly showed that class I antibodies (3B11 and 20G1) bind to the proregion of desmoglein-2, and that class II antibodies (7H9 and 19B9) recognize epitopes near the N-terminus of EC1 (Fig. 2). However, immunoblots of authentic desmoglein-2 from A431 cell extracts suggested that class II antibodies recognize desmoglein-2 in a proregion-dependent manner, even though the epitope clearly is not in the proregion since they do not recognize the GST-pro fusion protein. Table 1 compares the amino acid sequence of mature desmoglein-2 with that of several of the GST constructs used for mapping. Taken together, the data in Figures 2 and 3 suggest that class II antibodies recognize desmoglein-2 when Ala⁵⁰ is in a peptide bond, but not when Ala⁵⁰ is at the free N-terminus as it is in mature desmoglein-2, suggesting that the epitope for these antibodies is near the junction between the proregion and EC1.

Although antibodies that recognize an epitope in mature desmoglein-2 (6D8, DG3.1 [cytoplasmic epitope], and the class III antibodies) are expected to recognize each of the forms of desmoglein-2, they do not appear to bind to either component of the doublet recognized by the four proregion-dependent antibodies. This is likely because most of the desmoglein-2 is fully processed, and the small amount of immature protein is obscured by the antibody binding to the abundant mature form. To determine if

TABLE 1. AMINO ACID SEQUENCE OF GST FUSION PROTEINS AND MATURE DESMOGLEIN-2

<i>Protein</i>	<i>Sequence</i>	<i>Class II reactivity</i>
GST-proEC1N	HPHLVRQKR <u>A</u> WITAPVALRE... proregion EC1	+
GST-proEC1	HPHLVRQKR <u>A</u> WITAPVALRE... proregion EC1	+
GST-EC1N	TENLFQGAM <u>A</u> WITAPVALRE... GST vector EC1	+
Mature desmoglein-2	+NH ₃ <u>A</u> WITAPVALRE... EC1	—

The underscored residue in each sequence corresponds to Ala⁵⁰ of human desmoglein-2 (GenBank accession NM_001943). The nine upstream amino acids are either a portion of the proregion of desmoglein-2 or the amino acids encoded by the multiple cloning site of the pGST-Parallel vector. GST-proEC1N is comprised of GST fused to the N-terminal half of EC1. GST-proEC1 is comprised of GST fused to all of EC1. The N-terminal sequence of desmoglein-2 is also shown.

this is indeed the case, extracts of A431 cells were immunoprecipitated with anti-proregion antibodies and blotted back with DG3.1 (Fig. 3C). Class I and II antibodies pulled down the expected doublet, and this doublet was recognized by DG3.1 in an immunoblot, indicating that DG3.1 does recognize each form of desmoglein-2 and further verifying this doublet is a precursor for mature desmoglein-2.

To further verify that the bands recognized by the newly developed antibodies truly represent various forms of authentic desmoglein-2, we used shRNA to knock down desmoglein-2 expression in A431 cells and immunoblotted extracts of knock-down cells with each antibody. Immunoblotting with 6D8 showed that desmoglein-2 was significantly knocked down in cells expressing the shRNA compared to control cells (Fig. 3D, lanes 1 and 2). Indeed, each of the newly developed monoclonal antibodies detected less antigen in the knockdown cells than in the mock transfected cell. These data rule out the possibility that the doublet recognized by class I and II antibodies is due to non-specific binding by these four antibodies and confirm that they recognize authentic desmoglein-2. The class III antibodies also detected less desmoglein-2 in the knock-down cells than in the control cells.

Proregion-dependent antibodies recognize desmoglein-2 in the ER/Golgi

Immunofluorescence microscopy was used to investigate the localization of immature forms of desmoglein-2. Both of the class I antibodies that clearly mapped to the proregion, 3B11 and 20G1, showed strong nuclear staining, which is most likely nonspecific as there have been no reports of desmoglein-2 in the nucleus. In addition, previously characterized antibodies including 6D8 (from our lab) and DG3.1 (generated by Dr. Franke's lab) showed no nuclear signal, further indicating that the nuclear signal was non-specific. These antibodies also showed a diffuse perinuclear staining pattern indicative of endoplasmic reticulum (ER; Fig. 4A and B). However, the strong non-specific nuclear signal makes these two antibodies less useful for immunofluorescence experiments.

Class II antibodies strongly stained the perinuclear region of cells, corresponding to an ER/Golgi pattern, with no clear punctate staining indicative of desmosomes (Fig. 4C and D). These staining patterns support the idea that class II antibodies recognize proregion-containing forms of desmoglein-2 rather than mature desmoglein-2, and are consistent with

FIG. 4. Immunofluorescence staining with anti-desmoglein-2 antibodies. A431 cells grown on glass coverslips were fixed with Histochoice and stained with the various class I (A and B), class II (C and D), and class III (E and F) anti-desmoglein-2 antibodies using unpurified hybridoma supernatant. The previously described 6D8 antibody (also unpurified supernatant) was used as a control (G).

the previous data implicating these two antibodies as proregion dependent.

The class III antibodies that map to the C-terminal portion of EC1 stained cell-cell borders (Fig. 4E and F) in a pattern very similar to the previously characterized 6D8 antibody (Fig. 4G). Interestingly, 10D2 stained cell-cell borders in a pattern more punctate than is typically seen with anti-desmoglein antibodies, and resembled the staining pattern seen with anti-desmoplakin. In comparison, 6D8 stained cell-cell borders with an almost continuous pattern (see Fig. 4G). A possible explanation for the difference in staining seen with 10D2 and 6D8 is that 10D2 might recognize the subset of desmoglein-2 that is tightly incorporated into desmosomes and may correspond to desmoglein-2 that is participating in "hyper-adhesion,"⁽¹⁷⁾ while 6D8 recognizes all desmoglein-2, whether or not it is tightly incorporated into desmosomes.

To further address this question, we fractionated A431 cells into NP-40 soluble and insoluble fractions. The insoluble fraction is enriched in desmoglein-2 that is incorporated into desmosomes. The fractions were resolved by SDS-PAGE and immunoblotted with the panel of anti-desmoglein-2 antibodies. Each of the proregion-dependent antibodies preferentially recognized proteins in the soluble fraction, while the class III antibodies (10D2 and 13B11) and the previously characterized DG3.1 antibody recognized desmoglein-2 in both fractions (Fig. 5). Interestingly, 10D2 had a much greater preference for NP-40 insoluble desmoglein-2 than did 13B11 or DG3.1, providing further evidence that it prefers desmoglein that is tightly incorporated into desmosomes.

Desmoglein-2 containing the proregion can traffic to the plasma membrane

The proregion of classical cadherins is efficiently removed by furin proteases before the mature cadherin traffics to the cell surface.^(10,11) By mutating the cleavage site, it has been shown that proregion containing classic cadherins are able to traffic to the plasma membrane; however, they are not functional and are not present in any appreciable level under normal physiological conditions.⁽¹⁰⁾ Since we could readily observe immature forms of desmoglein-2 in cell extracts, we wanted to determine if proregion-containing desmoglein-2 is present on the cell surface. We surfaced biotinyl-

ated A431 cells, extracted them, and immunoprecipitated desmoglein-2, using class I, II, or III antibodies followed by blotting with horseradish peroxidase-labeled streptavidin. Desmoglein-2 recognized by 3B11 (class I) and 7H9 (class II) was biotinylated (Fig. 6A), providing evidence that proregion containing desmoglein-2 is found on the cell surface. As expected, 10D2 (class III) and 6D8 also immunoprecipitated biotinylated desmoglein-2.

Desmoglein-2 is cleaved extracellularly by ADAMs-10 and ADAMs-17, shedding the extracellular domain into the culture medium.⁽¹⁸⁾ To further confirm that unprocessed desmoglein-2 is present on the plasma membrane, we immunoprecipitated shed desmoglein-2 from the culture medium using the 6D8 monoclonal antibody (which recognizes the EC4 of desmoglein-2) and then blotted back with class I (3B11 and 20G1) or class II (7H9 and 19B9) antibodies, which recognize only desmoglein-2 that contains the proregion. 6D8 was used as a positive control and DG3.1 as a negative control, since this antibody recognizes the cytoplasmic domain of desmoglein-2, which is not included in the shed fragment. Each proregion-dependent antibody recognized the shed fragment of desmoglein-2 immunoprecipitated from culture medium, indicating that proregion containing forms of desmoglein-2 were in the culture supernatant (Fig. 6B).

Finally we performed immunofluorescence microscopy on cells that had not been fixed or permeabilized (Fig. 6C). Antibody 6D8 was used as a positive control because it recognizes EC4 of desmoglein-2 and should bind strongly to the cell surface. DG 3.1 was used as a negative control because it binds to the cytoplasmic domain of desmoglein-2 and should be completely negative if we have successfully protected the cells from permeabilization. The positive and negative controls indicated the experiment was performed successfully (Fig. 6C, panels a and b). Figure 6C panel c shows that the proregion-dependent antibody 19B9 binds to the surface of non-permeabilized A431 cells indicating that proregion-containing desmoglein-2 is present on the cell surface. Together these data suggest that the processing of desmoglein-2 may not be as rigorously controlled as the processing of classical cadherins.

Discussion

Desmogleins and desmocollins are the transmembrane components of the desmosome, a structure that provides

FIG. 5. Fractionation of cell extract. NP-40 soluble and NP-40 insoluble fractions of A431 cell extracts (50 μ g protein) were resolved by SDS-PAGE and immunoblotted with class I, II, and III antibodies using unpurified supernatant. DG3.1 was used as a control.

FIG. 6. A fraction of cell-surface desmoglein-2 retains the proregion. **(A)** A431 cells were surface labeled with biotin, and cell extract (250 μ g protein) was immunoprecipitated with class I, II, or III antibodies (300 μ L unpurified hybridoma supernatant). Anti-tubulin was used as a negative control for the immunoprecipitation, and 6D8, which should recognize all forms of desmoglein-2, was used as a positive control. Immunoprecipitation reactions were resolved by SDS-PAGE and blotted with horseradish peroxidase-labeled (HRP) streptavidin to identify biotinylated proteins. A431 cell extract was immunoblotted with 6D8 to identify desmoglein-2. **(B)** Culture supernatant from A431 cells was immunoprecipitated with class I or II antibodies. Monoclonal antibody 6D8 was used as a positive control, and DG3.1, which recognizes the cytoplasmic domain of desmoglein-2, was used as a negative control. Immunoprecipitations were resolved by SDS-PAGE and immunoblotted with 6D8 to identify the shed fragment of desmoglein-2. **(C)** Unfixed, non-permeabilized A431 cells grown on glass coverslips were stained for immunofluorescence microscopy with the 19B9 class II monoclonal-anti-desmoglein-2. 6D8 was used as a positive control, and DG3.1 was used as a negative control.

strength to tissues undergoing mechanical stress. The goal of the present study was to generate antibodies against the proregion and EC1 of desmoglein-2 to provide reagents that would be useful for investigating the trafficking and processing of this desmosomal cadherin. Commercial antibodies are not available against the proregion of desmoglein-2, thus cleavage of the proregion and processing of desmoglein-2 have not been examined. Epitope mapping shows that we have generated two antibodies, 3B11 and 20G1, that recognize epitopes within the proregion of desmoglein-2. These antibodies work well in immunoblotting but are not useful for immunofluorescence since they show significant non-specific nuclear staining. The second set of antibodies, 7H9 and 19B9, map to the extreme N-terminal portion of EC1 of desmoglein-2, but are dependent upon the proregion being present on the protein. The sequence Arg-Gln-Lys-Arg is found at the junction between the proregion and EC1 of desmoglein-2. This is thought to be the furin cleavage site.⁽¹⁹⁾ Furin is a subtilisin-like proprotein convertase, which belongs to a family of calcium-dependent endoproteases and has an important func-

tion in the proteolytic processing of precursor proteins.⁽²⁰⁾ Furin has been shown to be predominantly localized and proteolytically active in the *trans*-Golgi network.⁽²¹⁾ Immunofluorescence localization using 7H9 or 19B9 shows strong localization to the ER/Golgi, suggesting that these antibodies recognize desmoglein-2 that has not yet left this compartment.

Proregion-dependent anti-desmoglein-2 antibodies recognize a doublet. It is expected that proregion containing forms of a protein would migrate at a higher molecular weight than the mature protein, but in our case we found that the mature form of desmoglein-2 migrates at a molecular weight intermediately between the two bands in the doublet. This suggests that immature forms of desmoglein-2 may not be fully glycosylated and thus migrate at multiple molecular weights, depending on the extent of glycosylation. Alternatively, the proregion of desmoglein-2 may be cleaved in multiple steps, producing more than one proregion-containing band. The small size of the proregion of desmoglein-2 makes analysis of the cleavage products difficult.

Desmosomes are highly insoluble in nonionic detergents, such as Triton X-100, and proteins found in the insoluble fraction are thought to be the proteins that are incorporated into desmosomes. Solubility studies showed that the class III antibody 10D2 mainly recognizes protein in the insoluble fraction, and immunofluorescence showed that this antibody stains cells in a punctate pattern characteristic of desmoplakin staining. These results suggest that 10D2 recognizes an epitope that is specific to desmoglein-2 that is incorporated into desmosomes. One hypothesis that would explain this unusual specificity of the 10D2 antibody is that a conformational change occurs in desmoglein-2 when it is incorporated into the desmosome, as suggested by Garrod and Kimura.⁽¹⁷⁾ This would not be the first case of an antibody recognizing an epitope that is dependent on cadherin conformation. For example, GC4, a monoclonal antibody against N-cadherin, recognizes an epitope that includes residues K64, P65, D67, and Q70. However for this antibody to bind to N-cadherin Trp2, which is 30 Å away, must be docked into a hydrophobic pocket that is a structural feature of the cadherin.⁽²²⁾ This suggests that Trp2 imposes structural constraints on the GC4 epitope. Additional studies are required to determine if a conformational change does indeed occur in desmoglein-2 when it is tightly associated with a desmosome and the 10D2 antibody may be useful to further characterize desmoglein-2 in desmosomes.

The classic cadherins are synthesized as precursor proteins that must be proteolytically cleaved to generate functional, mature proteins.^(10,19) It has also been shown that even two extra amino acid residues on the N-terminus of the classic cadherins disrupts adhesive function.⁽²²⁾ Our study shows that processing of desmosomal cadherins may not be as tightly regulated as that of classic cadherins. Surface biotinylation and immunofluorescence localization in non-permeabilized cells showed that a significant amount of proregion containing desmoglein-2 is present on the cell surface. Biotinylation studies performed in our lab showed no evidence for immature E-cadherin at the cell surface (data not shown), suggesting that classic cadherins are more efficiently processed than desmoglein-2. The significance of the presence of proregion containing desmoglein-2 on the cell surface is not understood at this time. It is possible that the presence of the proregion does not impair desmoglein-2 function. However, this is unlikely, since immunofluorescence localization suggests that proregion-containing desmoglein-2 is diffusely localized on the cell surface and is not within the desmosomal structure.

In summary, we have produced a panel of monoclonal antibodies against desmoglein-2 including antibodies that recognize desmoglein-2 in a proregion-dependent manner. These antibodies are useful in immunofluorescence, immunoblotting, and immunoprecipitation. Further studies to document the trafficking of desmoglein-2 will increase our understanding of the role of desmosomal cadherins in cell-cell adhesion and may contribute to our understanding of how desmoglein-2 mutations alter its function in diseases such as ARVC.

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