

Stabilization of Plakoglobin and Enhanced Keratinocyte Cell-Cell Adhesion by Intracellular O-Glycosylation*

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O-Glycosylation modifies and regulates a variety of intracellular proteins. Plakoglobin, which functions in both cell-cell adhesion and signal transduction, is modified by O-glycosylation; however, the significance is unknown. To investigate the functional consequence of plakoglobin O-glycosylation, we cloned and overexpressed in keratinocytes murine O-GlcNAc transferase (mOGT). Over expression of mOGT in murine keratinocytes resulted in (i) glycosylation of plakoglobin and (ii) increased levels of plakoglobin due to post-translational stabilization of plakoglobin. Additionally, overexpression of mOGT in keratinocytes correlated with increased staining for cell-cell adhesion proteins and greater cell-cell adhesion. These observations suggest that O-glycosylation functions to regulate the post-translational stability of plakoglobin and keratinocyte cell-cell adhesion.

Plakoglobin is a component of (i) adherens junctions, linking E-cadherin to the actin binding protein α -catenin, and (ii) desmosomes, linking desmogleins to desmoplakin, plakophilin, and in turn, keratin intermediate filaments. The suggestion that the association of plakoglobin with the adherens junction nucleates the formation of desmosomes may explain why plakoglobin is a component of both adherens junctions and desmosomes (1, 2), whereas β -catenin is limited to adherens junctions. Phosphorylation regulates the adhesion (3) and signaling (4) functions of plakoglobin. Plakoglobin is also modified by intracellular O-glycosylation (5); however, the functional consequence of plakoglobin O-glycosylation is not known.

Intracellular protein O-glycosylation (6) modifies and regulates a variety of substrates including transcription factors, nuclear pore and cytoskeletal proteins, and enzymes (7). *N*-Acetylglucosamine (GlcNAc)² modification of serine and threonine is catalyzed by the enzyme UDP-*N*-acetylglucosamine-polypeptide β -*N*-acetylglucosaminyl transferase (O-GlcNAc transferase, OGT) (8), whereas, GlcNAc is removed by O-GlcNAc-selective *N*-acetyl- β -D-glucosaminidase (9). OGT is highly conserved across species including humans, rodents, and nematodes (10, 11).

To investigate the functional consequence of plakoglobin O-glycosylation, we cloned and expressed in murine keratinocytes the murine OGT. We demonstrate that increased O-glycosylation is associated with increased plakoglobin protein levels, increased post-translational

stability of plakoglobin, and greater keratinocyte cell-cell adhesion. Greater cell-cell adhesion in OGT overexpressing keratinocytes is likely because of both the increased amounts of and enhanced cell membrane localization of cell-cell adhesion proteins.

EXPERIMENTAL PROCEDURES

Generation of Murine OGT Expression Vectors—Oligonucleotide primers flanking the published coding region of the murine OGT cDNA (12) were employed in a polymerase chain reaction-based strategy to clone the full-length nucleocytoplasmic OGT cDNA from murine keratinocytes. The pCS2myc-mOGT expression vector was constructed by cloning the full-length mOGT into the expression vector pCS2 (13) with six tandem copies of the human myc epitope in-frame with the N terminus of mOGT (see Fig. 1A). The pTRE2myc-mOGT expression vector was constructed by subcloning myc-tagged mOGT from pCS2myc-mOGT into the BamHI and NotI sites of pTRE-2 (Clontech).

Cell Culture and Transfection—The murine keratinocyte cell line PAM212 was cultured in RPMI 1640 (Invitrogen, Inc.) supplemented with 10% fetal bovine serum. The pCS2myc-mOGT expression vector was transiently transfected into subconfluent PAM212 cells using Effectene (Qiagen, Inc.). Stably transfected inducible cultures were obtained by cotransfection of pTRE2myc-mOGT and pTet-on (Clontech) into PAM212 cells as above and selected with G418 (Invitrogen, Inc.) and hygromycin B (Roche Diagnostics). mOGT expression was induced in stably transfected cells with doxycycline (2 mg/ml) for 24h. Experiments described utilized the permanently transfected cells (referred to as OGT cells); however, similar increases in plakoglobin protein levels were obtained with multiple independent transient transfections.

Antibodies—Immunoblotting, immunoprecipitation, and immunostaining were performed with primary antibodies against plakoglobin, β -catenin, E-cadherin, desmoglein-1 (dsg1) (BD Biosciences, Inc.), α -catenin (Zymed Laboratories, Inc.), desmoplakin 1/2 (Abcam, Inc), plakophilin 1 (Santa Cruz Biotechnology, Inc.), O-GlcNAc (clone RL-2, Affinity Bioreagents, Inc.), O-GlcNAc (clone CTD110.6, Covance), lactate dehydrogenase V (Cortex Biochem, Inc.), actin (Calbiochem, Inc.), and myc (Clone 9B11, Cell Signaling Technology, Inc.).

Immunofluorescence and Confocal Microscopy—Cells grown on glass coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.25% Triton X-100, blocked in 2% bovine serum albumin, and incubated with primary antibodies in PBS, 2% bovine serum albumin for 1 h. After washing with PBS, cells were incubated for 1 h with Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch laboratory, Inc.), washed, and mounted. Images were analyzed using a Leica SP2 AOBs confocal microscope as previously described (14).

Immunoblotting and Immunoprecipitation—Monolayer cells grown to confluence were extracted in cell lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μ M E64, 100 μ M leupeptin, 10 μ M pepstatin, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 1 h with rotating and then centrifuged at 13,700 \times g for 15 min

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² The abbreviations used are: GlcNAc, *N*-acetylglucosamine; dsg1, desmoglein 1; OGT, O-GlcNAc transferase; mOGT, murine OGT; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PP2A, protein phosphatase 2A; MOPS, 4-morpholinopropanesulfonic acid.

at 4 °C. The supernatants were collected as detergent-soluble fractions. The pellets were washed twice with PBS, resuspended by incubation in urea lysis buffer (8 M urea, 4% CHAPS, 10 mM Tris-HCl, pH 7.4) for 1 h at 4 °C, and then centrifuged as above; the supernatant was used as the detergent-insoluble fraction. Samples were equally loaded on and separated by SDS-PAGE. For immunoprecipitation, detergent-soluble fractions were incubated with antibodies and recombinant protein G-Sepharose 4B conjugate beads (Zymed Laboratories, Inc.) with rotating for 16 h at 4 °C. Immunoprecipitates were washed three times with PBS, denatured with SDS sample buffer, and separated by SDS-PAGE. Immunoblotting was performed according to established protocols and developed by enhanced chemiluminescence (ECL) reaction (Amersham Biosciences). Signal intensity from the ECL reaction for each band was quantified with a GeneGnome scanner (Syngene Bio Imaging) using GeneSnap software.

Two-dimensional Gel Electrophoresis—Cell extracts (100 μ g) were prepared and separated in the first dimension using 13-cm pH 3–10, non-linear IPGphor strips (Amersham Biosciences, Inc) and in the second dimension by 10% SDS-PAGE followed by immunoblotting as described (14).

Protein Phosphatase 2A (PP2A) Treatment—Cell extracts were prepared in 100 mM NaCl, 20 mM MOPS, pH 7.5, 1 mg/ml bovine serum albumin, 60 mM β -mercaptoethanol, 10 μ M E64, 100 μ M leupeptin, 10 μ M pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μ M okadaic acid (okadaic acid was omitted from buffer for PP2A-treated extracts) by sonication for 5 s \times 2 on ice, followed by centrifugation at 14,000 rpm in a microcentrifuge, and supernatants were collected in 500- μ g protein aliquots. For samples treated with PP2A, 500- μ g protein extracts were treated with 0.1 units PP2A (Upstate, Inc.) for 30 min at 30 °C. For two-dimensional gel electrophoresis, samples were desalted on Centricon 30 spin filters (Amicon Bioseparations, Inc.) and resuspended in 8 M urea, 4% CHAPS, 10 mM Tris-HCl, pH 7.4. Dithiothreitol was added to a final concentration of 2.5 mM and IPG buffer (pH 4–7, linear, Amersham Biosciences) was added to a final concentration of 0.5%, and the samples were separated in the first dimension using 7-cm, pH 4–7, linear IPGphor strips (Amersham Biosciences, Inc) and in the second dimension by 10% SDS-PAGE followed by immunoblotting as described (14).

Dispase-based Dissociation Assay—A dispase-based dissociation assay was performed as described (15, 16). Briefly, cells grown to confluence in triplicate on 100-mm dishes were washed twice with PBS, incubated in 5 ml of dispase II (2.4 units/ml, Roche Diagnostics) at 37 °C for 1 h, and rocked back and forth 10 times on a ClayAdams nutator, and the number of fragments was counted.

[3 H]GlcNAc Radiolabeling—80% confluent cultures were incubated with D-[6- 3 H]glucosamine hydrochloride (Amersham Biosciences) at 1 μ Ci/ml in glucose-free RPMI 1640 with 10% fetal bovine serum for 16 h at 37 °C, washed 3 \times in PBS, and extracted in cell lysis buffer as above. Soluble fractions containing equal amounts of protein were subjected to immunoprecipitation, separated by SDS-PAGE, and transferred to nitrocellulose, and the radioactive signal was visualized on a Molecular Dynamics Storm 840 phosphorimager.

Galactosyltransferase Labeling—Galactosyltransferase labeling was as described (5, 17). Briefly, plakoglobin immunoprecipitation reactions were washed 6 times in buffer A (15 mM Tris, pH 7.4, 5 mM EDTA, 1 M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) and resuspended in 500 μ l of buffer B (50 mM Hepes, 5 mM MnCl₂, 10 mM galactose, 2% Triton X-100). The labeling reaction was initiated by adding 2.5 μ Ci of UDP-[6- 3 H]galactose (American Radiolabeled Chemicals) and 50 milliunits of galactosyltransferase (Sigma) and incubating at 37 °C for 1 h. The beads were washed four times with buffer A, resus-

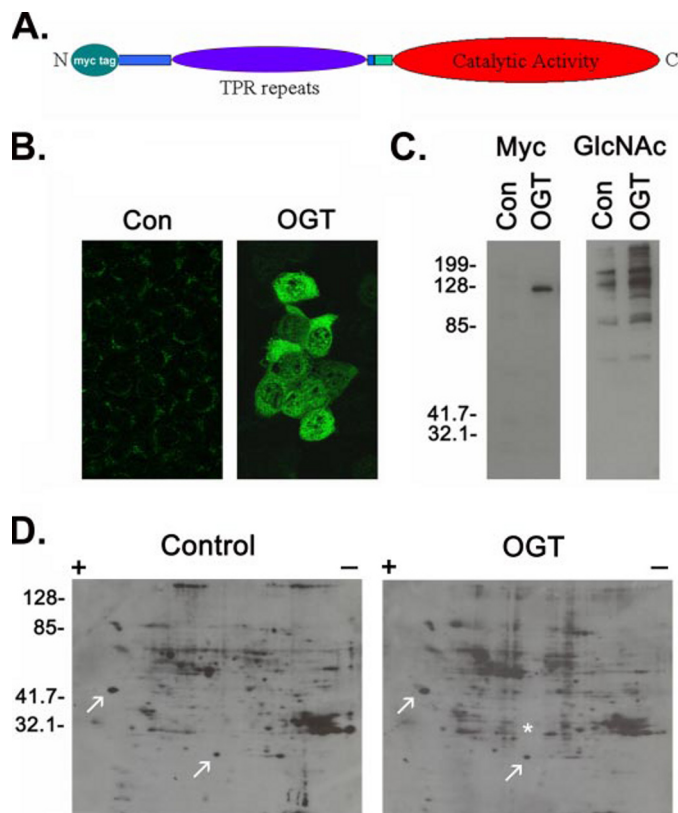


FIGURE 1. A, model depicting myc-tagged mOGT. B, The mOGT transgene is expressed in both the nuclei and cytoplasm of transfected cells. Confocal immunofluorescence microscopy using myc antibody detects myc-tagged expressed protein in OGT cells but not in non-transfected keratinocyte controls (Con). C, transfected cells express catalytically active mOGT. Cell extracts from control and OGT cells probed by immunoblot for mOGT transgene expression using myc antibody (Myc) and modification by *N*-acetylglucosamine using anti-GlcNAc antibody (GlcNAc). A single band of M_r ~120,000, corresponding to the expected M_r of the myc-tagged OGT protein, is detected in OGT cells. Greater reactivity to the GlcNAc specific antibody in OGT cells because of increased *O*-glycosylation demonstrates that the cloned OGT is catalytically active. D, immunoblot analysis using the GlcNAc-specific monoclonal antibody RL2 demonstrates increased GlcNAc modification in OGT cells. Cell extracts from control and OGT cells were separated by two-dimensional gel electrophoresis using 13-cm pH 3–10 non-linear IPGphor strips for the first dimension and 10% SDS-PAGE for the second dimension. The asterisk denotes additional GlcNAc modification of proteins detected in OGT cells not seen in controls. Arrows depict GlcNAc modification of proteins not altered in OGT cells (+, anode; -, cathode).

uspended in 20 μ l of SDS-PAGE sample buffer, incubated at 37 °C for 10 min, and subjected to SDS-PAGE followed by autoradiography.

Plakoglobin Half-life—The plakoglobin half-life was measured by treating confluent cells with the 20 mg/ml protein synthesis inhibitor cycloheximide (Sigma Aldrich) for 0, 2.5, 5.0, 7.5, and 10 h. Cells were harvested, and extracts were prepared as above. Soluble fractions containing equal amounts of protein (20 μ g) were subjected to SDS-PAGE and immunoblot with antibodies to plakoglobin and actin. Blots were developed by ECL and quantified as above using a GeneGnome scanner (Syngene Bio Imaging) and GeneSnap software.

RESULTS

The Cloned cDNA Encodes mOGT and Is Catalytically Active—Myc tagged OGT (Fig. 1A) localized to both the cytoplasm and nucleus (Fig. 1B) as expected for the described distribution of this intracellular enzyme (11). The expressed protein migrated on SDS-PAGE as a single band of 120,000 consistent with the predicted molecular weight for the full-length expressed OGT polypeptide fused to the 6-copy myc epitope tag (Fig. 1C). Increased reactivity with the GlcNAc-specific RL2 monoclonal antibody (18) was detected by immunoblot of OGT cell extracts

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compared with control cells demonstrating that the expressed myc-tagged protein was catalytically active (Fig. 1, C and D).

Plakoglobin Is a Substrate for mOGT—To demonstrate that the cloned cDNA encoded the enzyme that was in fact responsible for catalyzing the addition of GlcNAc to plakoglobin, plakoglobin was immunoprecipitated from control and OGT cell extracts, and the immunoprecipitation reactions were probed for plakoglobin and GlcNAc modification using anti-plakoglobin and anti-GlcNAc monoclonal antibodies, respectively (Fig. 2, A and B). Greater GlcNAc reactivity was detected in plakoglobin from the OGT cells using both the RL2 (Fig. 2A) and CTD110.6 (Fig. 2B) (19) GlcNAc-specific monoclonal antibodies. Quantitation of the signal intensity from replicates ($n = 3$) of the experiment shown in Fig. 2B demonstrated a 1.95 ± 0.03 -fold increase in GlcNAc immunoreactivity and a 1.5 ± 0.16 -fold increase in plakoglobin immunoreactivity in the plakoglobin immunoprecipitates from OGT versus control keratinocytes. Thus, the relative change in GlcNAc/plakoglobin between OGT and control cells was 1.3. The galactosyltransferase reaction catalyzes the transfer of [3 H]galactose (Gal) from UDP-

[3 H]Gal to terminal non-reducing GlcNAc. Using galactosyltransferase, plakoglobin from both control and OGT cells was labeled with [3 H]galactose; however, the signal was greater in OGT cells (Fig. 2C) compared with controls. As a further test, both control and OGT keratinocytes were incubated in the presence of [3 H]glucosamine. More tritiated sugar was detected in plakoglobin immunoprecipitates from OGT cells relative to controls (Fig. 2C).

When analyzed by two-dimensional gel electrophoresis, OGT cell extracts had more positively charged isoforms compared with controls (Fig. 2D). Removal of phosphate by treating control and OGT cell extracts with PP2A shifted the migration of plakoglobin toward the cathode. This suggests that the more positively charged isoforms in OGT cells are from the loss of phosphate likely because of the presence of the GlcNAc.

Increased Protein Levels of Plakoglobin Are Detected in OGT Cells—Interestingly, more plakoglobin was immunoprecipitated from OGT versus control cells, suggesting increased levels of plakoglobin in the OGT keratinocytes. This observation was confirmed by immunoblot analysis of plakoglobin in cell extracts; more plakoglobin was detected in OGT keratinocytes (Fig. 3A).

Expression of OGT Increases the Half-life of Plakoglobin—Direct modification of plakoglobin by O-glycosylation and the increased levels of plakoglobin in OGT cells suggested that O-glycosylation of plakoglobin might function to increase the post-translational stability of plakoglobin. To investigate the effects of O-glycosylation on the half-life of plakoglobin, control and OGT cells were treated with the protein synthesis inhibitor cycloheximide and the levels of plakoglobin as a function of time determined by immunoblot of whole cell extracts (Fig. 3, B–D). At 10 h, plakoglobin levels decreased to 30 versus 80% of starting levels in control versus OGT cells indicating that O-glycosylation of plakoglobin extended its half-life 2.7-fold. This effect was not a general effect on protein stability, as the half-life of actin in control and OGT cells did not markedly differ.

Cell-Cell Adhesion Is Increased in OGT Cells—Because plakoglobin functions in cell-cell adhesion as a component of both the keratinocyte adherens junction and desmosome, we next investigated whether OGT cells demonstrated greater cell-cell adhesion using the dispase assay (15). In this assay, cells are grown to confluence and floated off the plate as an intact sheet using dispase. The cell sheets are then rocked back and forth a set number of times on a nutator, which subjects the sheet to a shear stress. Greater cell-cell adhesion confers resistance to the shearing stress and results in decreased fragmentation of the cell sheet. The num-

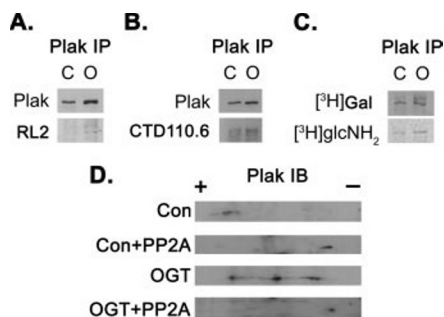


FIGURE 2. OGT modifies plakoglobin. A and B, plakoglobin (Plak) immunoprecipitates (IP) from control (C) and OGT cells (O) immunoblotted with plakoglobin- and GlcNAc-specific monoclonal antibodies, RL2, and CTD110.6. C, galactosyltransferase and glucosamine labeling. Plakoglobin was immunoprecipitated from control and OGT cells and radiolabeled with tritiated galactose using galactosyltransferase; the autoradiogram of samples was separated by SDS-PAGE ([3 H]Gal). Alternatively, plakoglobin was immunoprecipitated from control or OGT cells cultured in the presence of tritiated glucosamine, separated by SDS-PAGE, transferred to polyvinylidene difluoride, and scanned for tritiated N-acetylglucosamine modification ([3 H]glcNH $_2$) using a Molecular Dynamics phosphorimager. D, control (Con) and OGT extracts or control extracts treated with PP2A (Con+PP2A) and OGT extracts treated with PP2A (OGT+PP2A) were separated by two-dimensional gel electrophoresis using 7-cm pH 4–7 linear IPGphor strips in the first dimension and 10% SDS-PAGE for the second dimension and immunoblotted (IB) with plakoglobin antibodies. Additional positively charged isoforms of plakoglobin are present in OGT cells compared with controls. The more negatively charged isoforms present in both OGT and control cells are because of plakoglobin phosphorylation as treatment with PP2A results in an isoform mobility shift toward the cathode (+, anode; –, cathode).

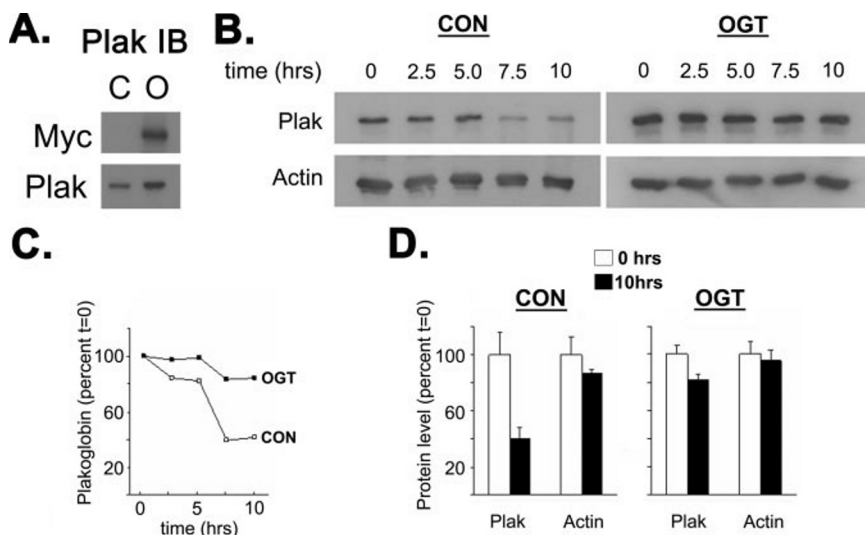


FIGURE 3. A, OGT increases plakoglobin levels. Control (C) and OGT (O) extracts separated by one-dimensional SDS-PAGE and immunoblotted with myc-tag and plakoglobin antibodies. B–D, GlcNAc-modified plakoglobin from OGT cells has a longer half-life ($t_{1/2}$). OGT and control (Con) cells were treated with the protein synthesis inhibitor cycloheximide, and the levels of plakoglobin were detected at the indicated times by immunoblot analysis using antibodies to plakoglobin. Blots were stripped and reprobbed with antibodies to actin as a loading control. B, representative experiment. C, plot of values from scanned gel as a function of time. D, quantitation of plakoglobin levels 10 h after inhibition of protein synthesis ($n = 3$, S.D. shown by error bars).

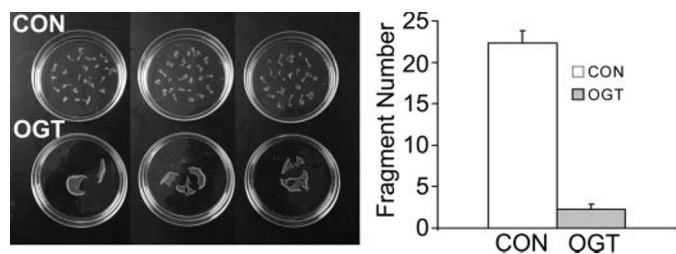


FIGURE 4. Cell-cell adhesion is increased in OGT cells. Confluent monolayer cultures of control (CON) and OGT keratinocytes were floated off the plates with dispase and subjected to shear force by rocking the cultures back and forth 10 times. Fewer (number of fragments = 2.3 ± 0.6) and larger fragments were generated from the OGT cells indicating greater cell-cell adhesion compared with controls (S.D. shown by error bars; $n = 3$).

number of resulting fragments yields a quantitative measure of cell-cell adhesion. When subjected to this assay, OGT cells formed far fewer and larger fragments per dish (number of fragments/dish = 2.3 ± 0.6) than control cells (number of fragments/dish = 22.3 ± 1.5) consistent with greater cell-cell adhesion in OGT cells (Fig. 4).

Plakoglobin Stabilization in OGT Cells Is Associated with Alterations in Adherens Junction and Desmosome Protein Localization—We next examined the distribution of E-cadherin and desmoglein-1 (dsg1) by confocal immunofluorescence microscopy (Fig. 5). In control cells, both punctuate cytoplasmic and membrane staining was observed for E-cadherin. Dsg1 staining in control cells was predominantly cytoplasmic, although a small amount of membrane staining could also be detected. In contrast, E-cadherin and dsg1 staining in OGT cells was predominantly localized to the cell membrane (Fig. 5A). Similarly, plakoglobin staining was both cytoplasmic and membrane in control keratinocytes, whereas greater plakoglobin membrane staining was observed in OGT cells. Furthermore, punctuate membrane staining for both plakoglobin and dsg1 was observed in the OGT cells, consistent with the staining pattern expected for desmosomes (Fig. 5B). OGT cells also demonstrated altered immunofluorescent staining for desmoplakin 1 and plakophilin 1, two additional desmosome components. Desmoplakin staining was punctuate and cytoplasmic in control cells; no membrane staining was observed. In contrast, membrane staining for desmoplakin was observed in OGT cells, although additional diffuse cytoplasmic staining was also present (Fig. 5C). Similarly, plakophilin staining in control cells was punctate and cytoplasmic, whereas punctate plakophilin membrane staining that colocalized with dsg1 was observed in OGT cells (Fig. 5D). Taken together, these results suggest increased membrane localization of both adherens junction and desmosome proteins in OGT overexpressing keratinocytes.

Increased Association of Plakoglobin with Both Adherens Junction and Desmosome Cadherins Is Observed in OGT Cells—We next examined the protein levels of adherens junction and desmosome components. Adherens junctions are soluble in non-ionic detergents such as Nonidet P-40, whereas desmosomes are insoluble in non-ionic detergents enabling us to use the detergent-insoluble fraction as a marker for desmosomes.

Control and OGT cells were separated into detergent-soluble and -insoluble fractions, and the fractions subjected to SDS-PAGE and immunoblotting using antibodies to adherens junction and desmosome proteins (Fig. 6). Similar levels of the adherens junction proteins E-cadherin, β -catenin, and α -catenin were detected in the detergent-soluble fraction of both control and OGT cells (Fig. 6A). In contrast, more plakoglobin was observed in the detergent-soluble fraction of OGT cells compared with controls.

Coimmunoprecipitation experiments from the detergent-soluble fraction were used to examine junctional protein-protein association.

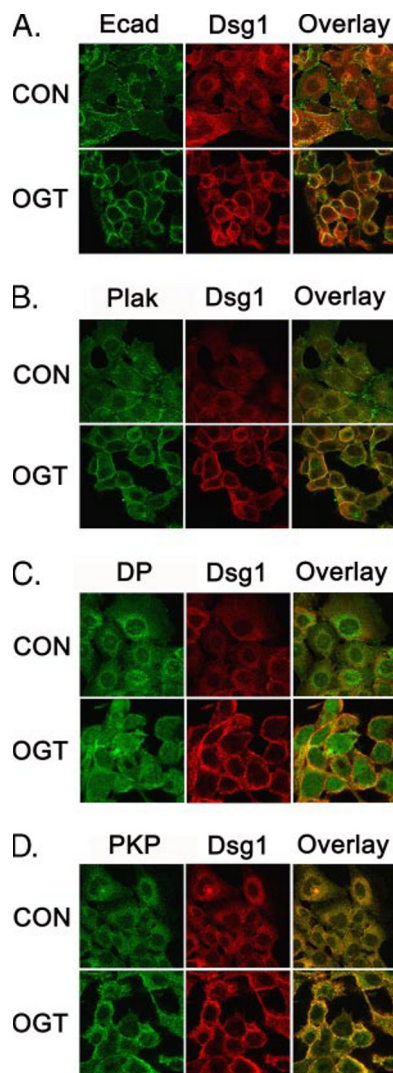


FIGURE 5. Increased membrane staining for cell adhesion junction proteins in OGT cells. Confocal immunofluorescence of control (CON) and OGT keratinocytes doubly stained with antibodies to E-cadherin (*Ecad*) and desmoglein 1 (*Dsg1*) (A), plakoglobin (*Plak*) and *dsg1* (B), desmoplakin (*DP*) and *dsg1* (C), and plakophilin (*PKP*) and *dsg1* (D). A, E-cadherin, *Dsg1*. In control cells, both punctuate cytoplasmic and membrane staining was observed for E-cadherin. *Dsg1* staining in control cells was predominantly cytoplasmic, although a small amount of membrane staining could also be detected. In contrast, E-cadherin and *Dsg1* staining in OGT cells was predominantly localized to the cell membrane B, plakoglobin, *Dsg1*. Plakoglobin staining was both cytoplasmic and membrane in control keratinocytes, whereas greater plakoglobin membrane staining was observed in OGT cells. Punctuate membrane staining of both plakoglobin and *Dsg1* in OGT cells is consistent with the staining pattern expected for desmosomes C, desmoplakin, *Dsg1*. Desmoplakin staining was punctuate and cytoplasmic in control cells; no membrane staining was observed. In contrast, membrane staining for desmoplakin was observed in OGT cells, although additional diffuse cytoplasmic staining was also present. D, plakophilin, *Dsg1*. Plakophilin staining in control cells was punctate and cytoplasmic, whereas punctate plakophilin membrane staining that colocalized with *Dsg1* was observed in OGT cells.

Equal amounts of E-cadherin were immunoprecipitated from the detergent-soluble fraction of control and OGT cells (Fig. 6B). No difference in the association of β -catenin with E-cadherin was identified, as similar amounts of β -catenin coimmunoprecipitated with E-cadherin from control and OGT cells. In contrast, more plakoglobin and α -catenin coimmunoprecipitated with E-cadherin from OGT cells.

Next, detergent-soluble extracts were subjected to immunoprecipitation with monoclonal antibodies to plakoglobin. As before, more plakoglobin was immunoprecipitated from the detergent-soluble fraction of OGT cells (Fig. 6C). More E-cadherin as well as the desmosome cadherin *dsg1* coimmunoprecipitated with plakoglobin in OGT cells

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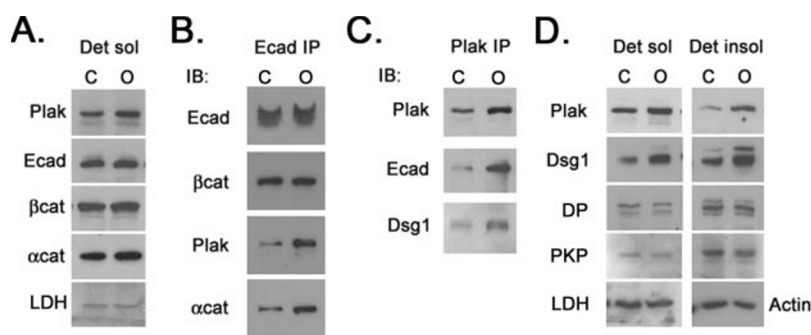


FIGURE 6. Levels of adherens junction and desmosome protein components in control and OGT cells. A, detergent-soluble (*Det sol*) fraction from control (C) and OGT (O) keratinocytes separated by SDS-PAGE and probed by Western blot for the adherens junction proteins plakoglobin (*Plak*), E-cadherin (*Ecad*), β -catenin (β *cat*), and α -catenin (α *cat*) or lactate dehydrogenase (*LDH*) as a loading control. B, E-cadherin was immunoprecipitated from the detergent-soluble fraction of control and OGT cells and immunoblotted (IB) with E-cadherin, β -catenin, plakoglobin, and α -catenin antibodies. C, plakoglobin immunoprecipitates (IP) from the detergent-soluble fraction of control and OGT cells were probed by immunoblot with antibodies to plakoglobin, E-cadherin, and dsg1. D, detergent-soluble and -insoluble (*Det insol*) fractions from control and OGT cells probed by immunoblot for the desmosome components plakoglobin, dsg1, desmoplakin (DP), and plakophilin (PKP) or lactate dehydrogenase and actin as loading controls for the detergent-soluble and -insoluble fractions, respectively.

indicating that O-glycosylation was associated with increased formation of plakoglobin cadherin complexes.

Both detergent-soluble and -insoluble fractions were probed by immunoblotting for desmosome proteins. Increased amounts of plakoglobin and dsg1 were observed in both the detergent-soluble and -insoluble fraction of OGT cells (Fig. 6D). The increased amount of the desmosome components plakoglobin and dsg1 in the detergent-insoluble fraction suggests increased desmosome assembly. Interestingly, similar increases in desmoplakin and plakophilin were not observed in the detergent-soluble and -insoluble fractions of OGT cells, despite the shift in the distribution of plakophilin and desmoplakin from cytoplasm to membrane observed by confocal immunofluorescence. These observations can be interpreted to suggest that although desmoplakin and plakophilin are moving toward the membrane, they have yet to be incorporated into fully formed desmosomes.

DISCUSSION

Taken together, the above observations demonstrate that increased post-translational stability of plakoglobin is a functional consequence of plakoglobin O-glycosylation. β -Catenin and plakoglobin are targeted for proteasome-mediated degradation; therefore, the observed increase in plakoglobin stability could be an indirect effect because O-glycosylation of the 26 S proteasome down-regulates proteasome-mediated protein degradation in rat kidney cells (20). Because β -catenin is also targeted to and degraded by the proteasome, greater amounts of β -catenin would be expected if the observed effect were because of regulation of proteasome activity. However, no difference in the levels of β -catenin were observed between OGT and control cells indicating that the effect of O-glycosylation on the post-translational stability of plakoglobin was unlikely the result of global changes in proteasome activity.

Direct modification of plakoglobin by O-glycosylation is likely responsible for increased stability to degradative pathways and therefore greater protein levels of plakoglobin in OGT cells. This interpretation is supported by several observations. Both plakoglobin O-glycosylation and plakoglobin levels were increased in OGT cells; however, the overall stoichiometry of plakoglobin O-glycosylation to plakoglobin was similar in OGT and control cells. Thus, each time a plakoglobin polypeptide is O-glycosylated, the amount of plakoglobin in the cell is increased by one molecule. Furthermore, the O-glycosylation site of plakoglobin has been mapped to threonine 14 (5) in the glycogen synthase kinase-3 phosphorylation consensus site. Threonine 14 may be phosphorylated by glycogen synthase kinase-3 as part of the mechanism for targeting plakoglobin to the proteasome for degradation or alternatively O-glycosylated resulting in increased stability. O-Gly-

cosylation and phosphorylation have been reported to alternately modify the same residue in other proteins (21).

Driving overexpression of OGT clearly results in the modification of numerous proteins as evidenced by the enhanced GlcNAc immunoreactivity observed on extracts separated by two-dimensional electrophoresis. It may be premature to suggest that modification of plakoglobin is the driving force for the observed increase in cell-cell adhesion, because O-glycosylation may modify a variety of proteins involved in adhesion. For example, keratins have been shown to be modified by O-glycosylation (22), and enhanced GlcNAc immunoreactivity was observed in the region of the two-dimensional gels to which keratins localize (Fig. 1D). Despite this, increased cell-cell adhesion resulted from driving overexpression of OGT in keratinocytes.

Interestingly, although plakoglobin levels were increased, the levels of β -catenin, which also associates with E-cadherin and α -catenin, were not altered. Increased association of plakoglobin with E-cadherin and α -catenin was observed in OGT cells suggesting increased numbers of plakoglobin-based adherens junctions. Similarly, OGT cells demonstrated (i) increased association of plakoglobin with the desmosome cadherin dsg1 in plakoglobin immunoprecipitates from the detergent-soluble fraction, (ii) more plakoglobin and dsg1 in the detergent-insoluble fraction, and (iii) greater membrane localization of desmosome proteins plakoglobin, dsg1, desmoplakin, and plakophilin.

Because both adherens junction formation and plakoglobin are important for nucleation of desmosome formation (1, 2), stabilization of plakoglobin by O-glycosylation may drive the formation of plakoglobin-based adherens junctions, which then nucleate formation of desmosomes, resulting in the observed increase in cell-cell adhesion.

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