Plakoglobin Is O-Glycosylated Close to the N-terminal Destruction Box*

Received for publication, February 6, 2003, and in revised form, June 27, 2003
Published, JBC Papers in Press, July 7, 2003, DOI 10.1074/jbc.M301346200

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Plakoglobin provides a key linkage in protein chains that connect desmosomal and classical cadherins to the cytoskeleton. It is also present in a significant cytosolic pool that has the capacity to impact on canonical Wnt signaling by competing for interaction with partner proteins of β-catenin. The closely related protein, β-catenin, is rapidly targeted for proteasomal degradation by phosphorylation of a “destruction box” within the N-terminal domain. Inhibition of this process forms the basis of Wnt signaling. This destruction box is also found in the N-terminal domain of plakoglobin. We report that plakoglobin is modified by the addition of O-GlcNAc at a single site in close proximity to the destruction box. O-GlcNAc modification has been proposed to counteract phosphorylation, provide protection from proteasomal degradation, mediate signal transduction, silence transcription, and regulate multimolecular protein assembly. This finding has potential implications for understanding the roles of plakoglobin.

Plakoglobin is a component of cell-cell adhesive junctions and at these sites forms a bridge between cadherins and cytoskeletal linker proteins (1–3). Its essential role in the maintenance of tissue integrity is revealed in the phenotypes of mice and humans with compromised plakoglobin activity. Most plakoglobin null mice die around embryonic day 15 (E15) with ruptured hearts and, those that survive longer (E18) show severe epidermal blistering and sloughing (4, 5). Likewise, mutations in the human plakoglobin gene that result in deletion of the C-terminal domain cause arrhythmogenic right ventricular cardiomyopathy, palmoplantar keratoderma, and wooly hair (6).

Plakoglobin is found in at least three types of cell junctions. In epithelial adherens junctions it connects classical cadherins to actin filaments via α-catenin. In the rare complexus adhaerentes junctions of endothelial cells it binds VE-cadherin to intermediate filaments via desmplakins I and II (7, 8). In desmosomes it engages in multiple lateral and vertical protein complexes: connecting desmplakins to the N termini of desmoplakin I and II, binding to the C terminus of desmocollin a, and associating with plakophilins, to promote cytoskeletal tethering and the lateral clustering of desmosomal cadherins (9–13).

In addition to these structural roles, plakoglobin recruits several kinase and phosphatase receptors including c-erbB-2, epidermal growth factor receptor, and LAR and associates with the breast antigen, MUC-1 (14–19).

As detailed above, plakoglobin engages in multiple mutually exclusive protein-protein interactions. However, little is understood about factors that regulate the selective assembly of these protein complexes. In the closely related protein, β-catenin, phosphorylation plays a critical role. Phosphorylations of the β-catenin ARM repeat domain decrease its positive charge leading to partner repulsion. Conversely, phosphorylation of partner proteins enhances their negative charge and promotes interaction (20, 21). Although most partner proteins interact with the central ARM repeat domain, the N-terminal domain of plakoglobin participates in binding α-catenin and promotes desmoglein association (11, 22). C-terminal domains of plakoglobin, β-catenin, and, their fly counterpart, Armadillo, interact with their respective N-terminal domains and/or ARM repeats and thus provide additional regulatory mechanisms by occupying and/or occluding the central binding groove (23–26).

In addition to their roles in cell junctions, plakoglobin and β-catenin participate in Wnt signal transduction pathways that pattern tissues and are deregulated in several forms of human cancer (27–32). The canonical Wnt signaling pathway operates by disabling a protein complex that phosphorylates a series of serines within the β-catenin N-terminal domain referred to as the destruction box (30, 33–36). Wnt inhibition of this phosphorylation cascade prevents subsequent ubiquitination and proteasomal degradation of cytosolic β-catenin. Once stabilized, β-catenin shuttles to and from the nucleus (37), regulating the activity of Lef/Tcf transcription factors (for references and current model see Footnote 1).

The N- and C- terminal domains of plakoglobin and β-catenin are extremely divergent, but the destruction box is found in both proteins (see Fig. 6). Plakoglobin is up-regulated by Wnt-1 and has the capacity to impinge on β-catenin signaling by binding to and competitively sequestering elements of the degradation machinery and Tcf/LEF transcriptional partner proteins (27, 31, 32, 38–51). Given the presence of this destruction box within the plakoglobin N-terminal domain and the critical role played by this region in the formation of several junctional protein complexes, an understanding of its post-translational modification is likely to be central to comprehending the cellular roles of this protein. In seeking to explore these issues, we discovered a site for O-GlcNAcylation on the N terminus of plakoglobin that lies intriguingly close to the destruction box. This modification has the potential to add an additional level of control to the protein stability, junctional and cytoskeletal as-

* This work was supported by National Institutes of Health GM47429 (to P. C.) and a New York State Health Research Science Board fellowship (to S. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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FIG. 1. Diagrammatic representation of plakoglobin and mutant constructs. Plakoglobin comprises 12 central 42-amino acid ARM repeats flanked by non-repetitive N-terminal (head) and C-terminal (tail) domains. Myc (m) and FLAG (f)-tagged mutant constructs are shown as lines with numbers indicating the initial and final amino acids of the human plakoglobin (GenBank™ Z68228) encoded by each construct. X indicates the positions of serines (S)/threonines (T) mutated to alanine (A).

EXPERIMENTAL PROCEDURES

Constructs—The following myc-tagged plakoglobin constructs in the p1637 vector (53) have been described previously: ΔN, ΔN-2, ΔN-6, ΔN-9, ΔS-C, ΔC-1, ΔC-N, (Fig. 1) (38, 54). ΔN32 and ΔN40 encoding FLAG-tagged mutants deleted in the N-terminal 32 and 40 amino acids, respectively, were generated as described for the full-length FLAG-tagged plakoglobin (PG) (38) by substituting FLAG-ΔN32F and FLAG-ΔN40F as the forward oligos. To generate serine/threonine, Ser/Thr, point mutants, complementary forward and reverse 24-base primers were designed comprising sequence flanking a three-base mutagenesis converting Ser/Thr codons to alanine (Ala) (Table I). Two sets of PCR reactions were done using 50 ng of hPG2.1, GenBank™ number Z68228 (55), as template and standard PCR conditions to generate overlapping fragments of plakoglobin that spanned and incorporated each mutant codon. These fragments were generated using either forward primer FLAG-120F, comprising a 3-base spacer, and reverse primer PG11RI, comprising a 3-bp spacer, or, a reverse primer 489R, comprising a 3-bp spacer, and a forward primer 37746 for galactosyltransferase (Gal-T) labeling and PNGase F analysis were carried out essentially as described (57). After immunoprecipitation, beads were washed six times in wash buffer (15 mM Tris, pH 7.4, 5 mM EDTA, 1 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100) and twice in Tris-buffered saline (TBS) for 1 h. Released saccharides were desalted by centrifugation at 12,000 × g for 5 min. Immunoprecipitation of plakoglobin was carried out with the following antibodies, mouse anti-FLAG (Sigma), 9E10 mouse anti-myc, mouse anti-plakoglobin 5172, rabbit anti-plakoglobin N terminus H-80 (Santa Cruz Biotechnology, Santa Cruz, CA), and guinea pig anti-plakoglobin 5f and 5l (1, 11). β-Catenin was immunoprecipitated using mouse anti-β-catenin (Transduction Labs, San Diego, CA). Coimmunoprecipitations were carried out using 10 μl of guinea pig antisera directed against desmoglein or desmocollin, as described (11) or anti-mouse E-cadherin (Transduction Labs). 10 μg of rabbit anti-mouse antibody (ICN Biomedicals Inc., Aurora, OH) was added to all immunoprecipitations using mouse antibodies. For steady state metabolic labeling, 24 h after transfection the cells were incubated for 12 h in 1 ml of methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 1% diazoylated fetal calf serum and 150 μCi/ml Tran35S-label labeling reagent (ICN Biomedicals Inc.). For pulse-chase experiments cells were labeled for 15 min with 50 μCi/ml Tran35S-label labeling reagent then rinsed and chased in Dulbecco’s modified Eagle’s medium containing 20 μM methionine. Primary mouse keratinocytes were prepared and maintained as described (38). Human HaCaT keratinocytes were maintained in KGM (Clonetics, Walkerville, MD) (56), human mammary epithelial cells MCF7, and vulval carcinoma A-431 cells were maintained in Dulbecco’s modified Eagle’s medium. Plakoglobin and β-catenin were immunoprecipitated from 100-mm dishes of cultured cells or from 50-30 μm cryosections of bovine snout epidermal tissue. These cells and tissue sources were lysed and processed as described above.

Cell Fractionation—Snout proteins were extracted sequentially in 1 ml of saponin buffer (0.01% saponin, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, and 10 μg of aprotinin), followed by 1 ml of Triton X-100 buffer (1% Triton X-100, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, and 10 μg of aprotinin) and then 200 μl of SDS buffer (1% SDS, 25 mM Tris, 3 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin). Extracts were centrifuged for 2 h at 100,000 × g. Equal amounts of plakoglobin were added to a final volume of 2 ml in buffer containing SDS at a final concentration of 0.1% and Triton X-100 at 1% prior to immunoprecipitation.

Galactosyltransferase (Gal-T) Labeling—Galactosyltransferase labeling and PNGase F analysis were carried out essentially as described (57). After immunoprecipitation, beads were washed six times in wash buffer (15 mM Tris, pH 7.4, 5 mM EDTA, 1 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) and twice in Tris-buffered saline, pelleted beads were resuspended in 500 μl of galactosyltransferase labeling buffer (50 mM Hepes, pH 7.4, 5 mM MnCl2, 10 mM galactose, 2% Triton X-100). The labeling was conducted under saturated conditions for galactosyltransferase (50 milliunits, Sigma) and UDP-[6-3H]galactose (ICN Biomedicals Inc., Aurora, OH) at 30°C for 1 h. After labeling, the beads were washed twice with Tris-buffered saline to remove unincorporated radiolabel prior to analysis by SDS-PAGE and fluorography.

Alkali-induced β-elimination was performed as described by Roquemore et al. (63). Briefly, after labeling and SDS-PAGE analysis, the proteins bound to band corresponding to plakoglobin were eluted, rinsed in water, and incubated in β-elimination buffer (1 mM NaBH4, 0.1 mM NaOH) for 24 h at 37°C. After 1 h on ice, the β-elimination reaction was stopped by addition of 4 μl ice-cold acetic acid until the pH dropped down between 6 and 7. Released saccharides were desalted by passage over Dowex 50-X8 (H+ form) and subjected to size fractionation on a Superdex peptide fast protein liquid chromatography column (Amersham Biosciences). The column was chromatographed at 0.5 ml/min in Milli Q water and was calibrated with partially hydrolyzed dextran standards and detected using an in-line Rainin refractive index monitor.
cytoplasmic because of lack of cell junctions in this cell line (11). In contrast, cultured keratinocytes, MCF-7, and A431 cells proliferate extensively and the majority of plakoglobin is localized at cell junctions (38). Sequential fractionation of snout plakoglobin showed the presence of the modified form in cytosolic (saponin) cadherin-bound (Triton X-100) and, to a lesser extent, in junctional (SDS) extracts (Fig. 2D).

**O-Glycosylation of Plakoglobin**

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**Plakoglobin Is Glycosylated**—Plakoglobin and β-catenin share over 65% sequence identity within their central ARM domains but diverge considerably in the sequence of their N-terminal domains, with the exception of a series of highly conserved sites for phosphorylation by casein kinase I and GSK-3 (55, 58, 59). During a sequence alignment of these regions, we noticed that plakoglobin, but not β-catenin, contained a sequence that is frequently modified by O-GlcNAcylation on many proteins (60, 61). O-GlcNAc monosaccharide modifications occur in a large number of nucleocytoplasmic proteins. In *vivo* these sugars are dynamically transferred from UDP-GlcNAc, the final product of the hexosamine pathway, to serines and threonines by the enzyme O-GlcNAc transferase and removed by the actions of the essential enzyme O-GlcNACase (52). To determine whether plakoglobin and β-catenin contain O-GlcNAc moieties, we transfected 293T cells with full-length plakoglobin or β-catenin cDNAs, recovered the expressed proteins by immunoprecipitation, and performed the Gal-T assay (Fig. 2A). In this assay, Gal-T covalently labels protein-bound sugar by transferring a galactose (Gal) from UDP-[3H]galactose on the GlcNAc modification. The products subjected to SDS-PAGE, and subjected to autoradiography to confirm the presence of [3H]galactose on the GlcNAc modification. The Coomassie Blue band corresponding to the position of plakoglobin was excised and subjected to alkaline β-elimination. The [3H]-labeled oligosaccharides released by this process were fractionated by chromatography. A single peak was observed that comigrated with the unlabeled disaccharide standard indicating the presence of [3H]Galβ1,4GlcNAcitol, the expected disaccharide product formed when galactose is added to GlcNAc residues during the Gal-T assay and released by alkaline β-elimination (Fig. 3). These results show that plakoglobin contains residues of N-acetylglucosamine that are O-glycosidically linked. To measure the amount of O-GlcNAc on plakoglobin, immunoprecipitates were subjected to the Gal-T assay, the products subjected to SDS-PAGE and stained with Coomassie Blue, and the amount of protein estimated by comparison to a series of bovine serum albumin standards. Gel slices containing the plakoglobin band were subjected to β-elimination and the moles of O-GlcNAc were estimated by counting the released [3H]galactose. Approximately 6% of plakoglobin is modified by O-GlcNAc. This, however, is likely to be an underestimate as the procedure is dependent upon the accessibility of the O-GlcNAc residue for further modification by Gal-T and does not take into account the potential dynamic nature of such a modification.

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**O-Glycosylation on the N-terminal Regulatory Domain**—To determine the site of O-GlcNAc modification, the

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products of deletion constructs of plakoglobin (Fig. 1) were examined (11). Plakoglobin comprises a central block of 12 highly conserved ARM repeats that provide specific binding sites for many different partner proteins flanked by non-repetitive N- and C-terminal domains (64). Deletion of the C-terminal domain and the ARM repeats did not abolish the Gal-T labeling, showing that O-linked GlcNAc residues are present in the N-terminal “head” domain (Fig. 4A). To determine whether the ARM repeats and C-terminal domain contains further modified residues, reverse deletion constructs were made (11). However, removal of the head domain of plakoglobin abolished Gal-T labeling (Fig. 4B). From this we conclude that the head domain contains all of the O-GlcNAc modified residues.

To further define the site(s) of modification within the head domain, we investigated three further deletion constructs. ΔN80, which lacks sequences encoding the first 80 amino acids, had been made previously for transgenic studies (38). No Gal-T labeling was observed on the product of this construct, thus defining the site of O-GlcNAcylation as within the first 80 amino acids (Fig. 4C, lane 3). This region contains a consensus site for GlcNAc modification (PSVSS) between residues 35 and 39 (60, 61). To determine whether this motif is modified, we generated two further constructs in which this sequence was retained (ΔN32) or deleted (ΔN40). However, Gal-T labeling of the products of both of these constructs was abolished (Fig. 4C, lanes 4 and 5) indicating that residues PSVSS do not constitute a site of glycosylation, and, that the site(s) of O-GlcNAc modification must lie within the first 32 amino acids of plakoglobin.

Plakoglobin Contains a Single Site of O-GlcNAc Modification at Thr-14 in Close Proximity to the GSK-3β consensus Site Destruction Box—The first 32 amino acids of plakoglobin contain the three GSK-3β consensus sites that correspond to the destruction box of β-catenin (Fig. 6) (65). A total of six threonines and serines are found within this stretch of plakoglobin sequence, five of which are conserved in β-catenin and in this protein are mutated in a large number of tumors (66). The O-GlcNAc modification could be readily detected in the product of the N-terminal domain construct (Δ1-C, Fig. 4A, lane 2, and Fig. 5A, lane 1) and was abolished by mutation of all of the first six serine/threonines within this region confirming that one or more of them can be glycosylated (Fig. 5A, lane 2). As GlcNAc modification has been reported to prevent proteolysis of several proteins by blocking key sites of phosphorylation (67, 68) we first investigated the consequences of mutating Ser-28, which corresponds to the Ser-37 site of phosphorylation in β-catenin that is essential for ubiquitination (65, 69). The product of S28A, which had been created for other purposes within the full-length clone, however, showed no reduction in Gal-T labeling (Fig. 5A, lane 10). We therefore mutated each of the remaining five candidate serine/threonines individually to alanine within the head domain construct and looked for loss of the Gal-T labeling on their mutant products. The products containing T19A, T21A, S24A, and T32A mutations continued to be labeled by Gal-T (Fig. 5A, lanes 4–7). However labeling of the T19A mutant was reduced (Fig. 5A, lane 4) and labeling of the T14A mutant was abolished (Fig. 5A, lane 3). Intriguingly, the T14A mutant did not migrate as fast as the mutant containing all six Ser/Thr-Ala mutations, which migrates significantly faster than the wild-type head domain. This suggests that further post-translational modifications of the first six

Fig. 2. [3H]Galactose-labeled plakoglobin and β-catenin. A, autoradiogram of immunoprecipitates of 293T cell lysates transfected with PG, empty vector, or β-catenin (β-cat) labeled by the transfer of [3H]galactose (Gal-T) or metabolically labeled by [35S]methionine (35S). Plakoglobin (lane 1) but not vector (lane 2) nor β-catenin (lane 3) immunoprecipitates label the Gal-T reaction. Plakoglobin (B) or β-catenin (C) immunoprecipitated from bovine snout and cell lines labeled by the transfer of [3H]galactose (Gal-T) or detected by Western blotting with the appropriate antibodies as indicated. D, immunoprecipitates from biochemical fractions of snout epidermis normalized for plakoglobin (PG) or β-catenin (β-cat) immunoprecipitated from bovine snout and cell lines labeled by the transfer of [3H]galactose (Gal-T) or detected by Western blotting as indicated.

Fig. 3. Identification of the products of β-elimination of [3H]galactose-labeled plakoglobin. The saccharide released from SDS-PAGE bands containing immunoprecipitated plakoglobin (squares) or an equivalent blank area of gel (circles) was analyzed by Superdex peptide fast protein liquid chromatography as described under “Experimental Procedures.” Diamonds indicate the positions of dextran size standards.
serine/threonine residues occur in addition to O-GlcNAcylation. To further test the significance of our deletion/mutation mapping we introduced T14A into full-length plakoglobin (PGT14A) and demonstrated that the product of this construct was not glycosylated (Fig. 5B). Thr-14 is one of several sites within the N-terminal domain predicted to be modified by O-GlcNAcylation. This residue is embedded within sequence EQPIKVTEWQQ containing several features that are commonly found at other sites of O-GlcNAcylation (Fig. 6) (60, 61). Specifically, Thr-14 is preceded by valine (Val-13), lies close to a proline (Pro-10), and neighbors a region of high serine/threonine density. An alignment of the N-terminal sequences of plakoglobin, β-catenin, and Armadillo shows that Thr-14 occupies the equivalent position to Ser-23 in β-catenin and possibly Ser-27 in Armadillo (55, 58, 59, 70). The sequence immediately following these residues is conserved in all three proteins notably in the WQQ motif followed by the highly conserved destruction box. To investigate if O-GlcNAcylation is required for the initial stages of junctional assembly we expressed full-length plakoglobin with and without the T14A mutation (PGT14A) and investigated its ability to bind to its cadherin partners in co-immunoprecipitation assays. Wild-type plakoglobin bound to desmoglein and was glycosylated. PGT14A plakoglobin binds equally well as wild-type plakoglobin to desmoglein 1, desmocollin 1a, and E-cadherin (Fig. 7A). Therefore, prevention of glycosylation, or any other potential modification, by mutation of Thr-14 to an alanine residue appears to have no effect on the initial interaction of plakoglobin with adhesion proteins. The fact that other cell lines form cell junctions in the absence of appreciable levels of O-GlcNAcylation of plakoglobin suggest that this modification is unlikely to play a role in the assembly of simple epithelial desmosomes. Next we compared the stability of full-length plakoglobin with and without the T14A mutation expressed in 293T cells by pulse-chase analysis and found the T14A product to be slightly more stable (Fig. 7B). A similar increase has been noted for ERβ, which is also modified to a comparable extent (6.7%) (71), and in this protein O-GlcNAc appears to inhibit destabilizing phosphorylations (67).

DISCUSSION

The primary finding of this report is that a proportion of plakoglobin is modified by the addition of a single O-GlcNAc residue on its N-terminal domain. This finding is based on the transfer of [3H]galactose from UDP-[3H]galactose to an N-terminal N-acetylglucosamine moiety present on the plakoglobin polypeptide by galactosyltransferase, the susceptibility of this galactosylated sugar to chemical cleavage by alkaline β-elimination, and its identification by Superdex gel filtration chromatography. A systematic mutational approach revealed a single site of modification on the first threonine residue (Thr-14) of the N-terminal domain. Thus, plakoglobin should be considered as a glycoprotein as well as a phosphoprotein and its
functions are likely to be regulated by both modifications. Modification of cytoplasmic and nuclear proteins by O-GlcNAcylation of serine or threonine residues has been found on nearly 100 eukaryotic proteins and the importance of this modification is attested to by the fact that β-N-acetylglucosaminyltransferase, the enzyme responsible for O-GlcNAc addition, is essential for ES cell viability (72, 73). Several observations support the concept that O-GlcNAc modifications play a regulatory role similar to that of phosphorylation (74). O-GlcNAcylation is dynamic, with the sugar moity turning over more rapidly than the protein and O-GlcNAc levels changing rapidly in response to external stimuli (75, 76). Almost all O-GlcNAc-modified proteins are phosphoproteins. Of potential significance for plakoglobin, β-catenin, and Armadillo proteins, O-GlcNAc sites often correspond to those used by proline-directed kinases, such as GSK-3β (61, 67, 77–79). O-GlcNAc is proposed to participate in signal transduction either by providing proteins with a tertiary phosphorylation switch (phosphorylated, O-GlcNAcylated, unmodified) (74, 80, 81). In addition to signaling, O-GlcNAcylation is implicated in several other roles that have potential significance for the functions of plakoglobin, such as silencing transcription (82, 83), acting as a nutritional sensor in the regulation of cell growth, and regulating protein conformation, multimeric protein assembly, and stability (52, 67, 68, 84, 85).

Given the well documented role of O-GlcNAc modification in protecting estrogen receptor and Sp1 from proteasomal degradation we were particularly intrigued by the site of O-GlcNAcylation on plakoglobin (67, 68). Thr-14 lies adjacent to the putative destruction box, a stretch of sequence that is highly conserved in β-catenin and IκB and in these proteins critically determines their stability and signaling functions (65). β-Catenin stability is tightly regulated by phosphorylation of Ser-45 by casein kinase I and sequential phosphorylation of Thr-41, Ser-37, Ser-33, Ser-29, and Ser-23 by GSK-3β (34–36, 86). This phosphorylation promotes interaction with the F-box protein β-TrCP leading to ubiquitination and proteasomal degradation. Plakoglobin contains this highly conserved destruction box, can be phosphorylated by GSK-3β, and, associates with several elements of the destruction machinery including axin (46), APC (50, 51), and the ubiquitin-proteasome system (42). Thr-14 and Thr-19 in plakoglobin occupy equivalent positions to Ser-23 and Ser-29 in β-catenin, which undergo GSK-3β-dependent phosphorylation and are mutated in hepatocellular and gastric tumors, respectively (86–88). ERβ and c-myc levels are also regulated by phosphorylations and experiments have strongly suggested that these are antagonized by O-GlcNAc modification (67, 81). Ser/Thr to alanine mutations in the O-GlcNAc site of ERβ result in a slight increase in protein stability, whereas phosphomimetic Ser/Thr to glycine mutants are more unstable. In the case of estrogen receptor, Sp1, c-myc, and RNA Pol II O-GlcNAc modification exerts its effects by antagonizing phosphorylation of the same site or of neighboring sites (67, 77–79, 82). The slight increase in stability of T14A plakoglobin mutants and the low levels of soluble plakoglobin seen in cell types where plakoglobin is not glycosylated may point to a similar mechanism. Although a direct role for plakoglobin in Wnt signaling is highly controversial, there is general agreement that plakoglobin can modulate β-catenin signaling positively by competitively binding elements of the destruction complex, and negatively via sequestration and inhibition of β-catenin signaling pathways that stabilize plakoglobin will suppress its responsiveness to external signals that operate through the destruction box and enhance its capacity to modulate β-catenin activity, providing an additional layer of control of several important signaling pathways that utilize β-catenin as a hub.

A second major proposed function for O-GlcNAc modification lies in silencing gene expression (82, 83). Many transcription factors, including estrogen receptor and Sp1, are modified and inactivated by O-GlcNAc (67, 77, 82). Recent studies have revealed that the enzyme responsible for O-GlcNAc addition, O-GlcNAc transferase, is recruited to gene promoters by Sin3A to co-repress transcription (83). These findings are intriguing.

**Fig. 6.** Alignment of the protein sequence of the head domains of plakoglobin, β-catenin, and Armadillo. Numbers indicate amino acid residues. Bold letters indicate amino acids that are conserved in two of the three sequences. Red highlights indicate the serine/threonine residues conserved in all three proteins within the destruction box, purple highlights indicate other serine/threonine residues. Large arrowheads point to Thr-14, the site of O-GlcNAcylation in plakoglobin. Arrowheads indicate the positions of deletions made within the head domain. Box indicates the α-catenin binding domain.

**Fig. 7.** Association with cadherins and stability of full-length plakoglobin and a PGT14A mutant. A, desmosomal (Dsg1, Dsc-1a) or classical cadherins (E-cad) were coexpressed in [35S]methionine metabolically labeled 293T cells together with full-length plakoglobin PG or a full-length mutant PGT14A and immunoprecipitated. PG and PGT14A co-precipitate equally well with each cadherin partner. Immunoprecipitates of desmoglein-bound plakoglobin and free plakoglobin are both labeled by the Gal-T assay (B). Immunoprecipitates of the products of PG and PGT14A expressed in 293T cells as detected by a 15-min pulse of [35S]methionine followed by chase times as indicated reveal no difference in stability.
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In light of the recent report that plakoglobin can form ternary complexes with β-catenin and Tcf and, in so doing, lowers the transcriptional activity of the complex (39).

A third important function of O-GlcNAcylation is in regulating the assembly and stability of reversible multimeric protein assemblies (67, 84, 85). In the case of secreted proteins it is generally accepted that glycosylation serves, at least in part, to stabilize proteins in otherwise unfavorable conformations. Thus, O-GlcNAc could act to stabilize and maintain a pool of incompletely folded plakoglobin, held in reserve for future assembly of its many protein partners, and/or enhance intramolecular protein folding. As detailed in the Introduction, plakoglobin engages in multiple mutually exclusive protein complexes. Several desmosomal components, including plakoglobin, bind to N-terminal sites in keratin 5 and other type II keratins in vitro (90, 91). Multiple sites of O-GlcNAcylation are present within the N-terminal domain of several keratins and are thus ideally positioned to modulate the assembly and stability of the desmosome–IF network (75, 92, 93). The effects of keratin mutants lacking these modifications have not been conduct in cell types where desmosome–IF association can be assessed, but similar O-GlcNAc sites in the N-terminal domains of neurofilaments have been strongly implicated in modulating their networking capabilities (84, 85). Although it is conceivable that O-GlcNAc modification could assist interactions among the desmosomal–IF proteins, we saw no deleterious effect of T14A mutations on plakoglobin–cadherin association in 293T cells. Moreover, lack of plakoglobin glycosylation did not prevent desmosome formation in several epithelial cell lines.

In conclusion we report that plakoglobin is modified by O-GlcNAc. The close proximity of the modified site (Thr-14) to the putative destruction box raises new questions about the functional significance of O-GlcNAc. β-Catenin has been reported to be O-glycosylated on the basis of its affinity for wheat germ agglutinin columns (94, 95). Our studies extend these observations to show that β-catenin and plakoglobin are differentially glycosylated in a cell context-specific manner. Although the significance of their differential glycosylation remains to be determined, it suggests new potential mechanisms by which the signaling functions of ARM proteins can be regulated.

Acknowledgments—We thank Catherine Fournier and Emmanuelle Charpentier for constructing S28A ΔN40 and ΔN32, Lauren Browne for technical assistance, and Irina Pechenkina for editorial assistance.

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