Density-dependent Lectin–Glycan Interactions as a Paradigm for Conditional Regulation by Posttranslational Modifications*

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Mice with null mutations in specific Golgi glycosyltransferases show evidence of glycan compensation where missing carbohydrate epitopes are found on biosynthetically related structures. Repetitive saccharide sequences within the larger glycan structures are functional epitopes recognized by animal lectins. These studies provide the first in vivo support for the existence of a feedback system that maintains and regulates glycan epitope density in cells. Receptor regulation by lectin–glycan interactions and the Golgi provides a mechanism for the adaptation of cell surface receptors and solute transporters in response to environmental cues and intracellular signaling. We suggest that other posttranslational modification systems might have similar conditional features regulated by density-dependent ligand–epitope interactions. Molecular & Cellular Proteomics 12: 10.1074/mcp.R112.026989, 913–920, 2013.

Cells must integrate multiple inputs (i.e. metabolites, trophic factors, pathogens) in order to maintain systemic control under a range of conditions. It is well established that many of these adaptive mechanisms involve posttranslational modifications (PTMs) of proteins such as phosphorylation, acylation, methylation, glycosylation, and others. The modifying enzymes recognize short consensus sequences in target proteins and a high energy donor substrate. The latter are metabolites, and their concentrations can also regulate PTMs (1). PTMs can exert conformation and allosteric effects on a target protein (2). However, PTMs also create binding sites (epitopes) for other proteins, thereby recruiting signaling complexes to biologically relevant regions in the cell. Most cytokine receptors and solute transporters are co-translationally N-glycosylated at NXS/T (X ≠ P) sites in the endoplasmic reticum. Some of the Asn-(N)-glycans bind chaperones that promote protein folding, secretion, or degradation of misfolded proteins (3). On the cell surface, N-glycans can serve as ligands for animal lectins (galectin, siglec, and C-type lectins) (4) that regulate receptor clustering and dynamics, while phosphorylation at multiple PTM sites on the cytoplasmic tails of transmembrane receptors or transporters recruits adaptor complexes (5). Adaptor proteins are often structured as tandem domains that bind different and overlapping sets of PTMs, in which multivalency is a critical feature (6). Multivalent systems display partial redundancy that might buffer the mutational loss and gain of sites, thereby promoting the evolution of PTM networks.

Membrane microdomains such as ganglioside-rich lipid rafts, coated pits, cell junctions, and focal adhesions are dynamic and regulate the activity of receptors including glycoproteins. Here we describe lectin binding to transmembrane glycoproteins, which forms dynamic cross-linked “lattices” (7, 8). Lectin–glycan interactions occur widely and have been implicated in many systemic processes in mammals (9), but the molecular mechanisms remain poorly understood. We suggest that glycan epitope density is highly regulated and has a global impact on lectin-mediated regulation of receptors and transporters at the cell surface (10–12) (Fig. 1). In these important features, glycosylation can serve as a model for density-dependent ligand control of PTMs in general.

Glycan Density, Affinity Enhancement, and Cross-linking—Glycans on the surface of cells are present as multivalent epitopes at densities that are compatible with lectin binding and the formation of cross-linked lattices. The valency of epitopes on glycoprotein receptors depends on the structure of the N- and O-glycans, as well as on the number of glycan sites per molecule, and it controls the affinity for lectins and cross-linking dynamics (13, 14). The affinity (avidity) of lectins for multivalent glycan epitopes depends, in part, on the three-dimensional arrangement of the lectin binding sites. Lectins with multiple binding sites aligned to a matching array of glycan epitopes show large affinity enhancements as a result of simultaneous binding. For example, the binding of the
asialoglycoprotein receptor to the N-acetyllactosamine (gαlactose/H9252, or LacNAc) branches of a triantennary N-glycan is 106-fold greater in affinity relative to monovalent LacNAc (15). Man-binding protein, a C-type lectin in the col lectin family that functions in innate immunity, possesses multiple triple helical collagen-like arms and terminal trimeric carbohydrate-binding subunits with aligned binding sites. As a consequence, Man-binding lectin exhibits high affinity and specificity for multiple Man residues on the surfaces of pathogens (16).

Plant and animal lectins with binding sites on subunits oriented in different directions can also bind with high affinity to multivalent carbohydrates and glycoproteins. In such a case, lectin affinity increases with the number (valence) of glycan epitopes in a molecule or on a surface (glycan density) (17). An example is the binding of galectins, a family of β-galactose specific animal lectins, to asialofetuin, a globular glycoprotein possessing three N-linked triantennary chains and nine terminal LacNAc residues, which results in 50- to 80-fold enhanced affinity relative to monovalent LacNAc (18). A dramatic example is binding of the GalNAc-specific soybean agglutinin to a linear glycoprotein (mucin) possessing ~2300 GalNAc residues, which results in a 106-fold enhanced affinity relative to monovalent GalNAc (19). The high affinity of the mucin is due to the large number of its glycans. The binding of lectins to glycan arrays also shows increasing
affinity with increasing glycan epitope density (20). Affinity enhancement in these multivalent systems is believed to be primarily due to slower effective off-rates, although increases in on-rates may occur (20). In essence, a bound lectin is likely primarily due to slower effective off-rates, although increases in these multivalent systems is believed to be attachment with increasing glycan epitope density (20). Affinity enhancement with increasing glycan epitope density (20)

Lectins with multiple binding sites can also form two- and three-dimensional cross-linked complexes (lattices) with multivalent glycans (23). For example, the Man-specific lectin concanavalin A, in the presence of a mixture of two multivalent carbohydrates that differ in the number of Man epitopes, can bind and separate into distinct cross-linked lattices with each glycan (24). The structural basis for the formation of separate (homogeneous) cross-linked lattices has been shown to be crystal-packing interactions (25, 26). Lectin-mediated cross-linking interactions regulate the levels and activities of glycoprotein receptors and transporters on cells, as discussed below.

In vitro studies suggest that galectin-1, a symmetrical dimer, can form homogeneous lattices with glycoproteins in vivo. In contrast, in vitro studies indicate that galectin-3 is a mixture of monomers and pentamers in solution, and that the latter form disorganized heterogeneous lattices with multivalent carbohydrates (30). For example, galectin-3 binds but does not selectively aggregate CD45 on the surface of apoptosis-sensitive T cells, as does galectin-1 (31). In addition, galectin-3 cross-links different cytokine receptors on the surface of cells, slowing their mobility and loss to endosomes and enhancing cellular sensitivity to ligand-dependent signaling (32, 33). The galectin-3 lattice also regulates the dynamics of receptors in a context-dependent manner. For example, galectin-3 binding reduces GFP-tagged EGF receptor mobility in the lipid bilayer while preserving sensitivity to EGF, but it increases mobility in focal adhesions and promotes PI3K signaling (33, 34) (Table I). The bi-, tri-, and tetraantennary N-glycans display increasing LacNAc epitope densities and affinity for galectins, respectively (35). However, galectin-1 and -3 differ in their tolerance of additional modification to the LacNAc epitope, which might contribute to their distinct cross-linking activities and biological properties (36).

Epitope Density Maintenance in N-glycans—Lectins bind to epitopes within the larger glycan structure, suggesting that glycans might be grouped into equivalence classes by epitope number and used to compute the affinity of glycoproteins for lectins. Lau et al. (10) developed a model for the regulation of cytokine receptors at the surface of mammary tumor cells based on epitope density and galectin-3 binding (Fig 2A). Microheterogeneity at each NXS/T site results in a distribution of glycoforms for each glycoprotein receptor, and the various combinations can be grouped by affinity for ga-

| Receptor and transporter | Glycans (gene) | Lectins | Dynamics in | Phenotype | Reference |
|-------------------------|--------------|---------|-------------|-----------|-----------|
| T cell receptor         | N-(Mgat5)²   | Gal-3   | Immune synapse (+) | Autoimmunity | (8)       |
| CTLA-4                  | N-(Mgat5)²   | Gal-3   | Membrane-endo | Autoimmunity | (10, 52)  |
| CD45 phosphatase        | N- and O-    | Gal-1, -3 | Membrane (− and +) | T cell activation | (29, 64) |
| K⁺ channel Kv1.3        | N-branching⁶ | ND      | Membrane-endo | Many cells | (65)      |
| EGFR and TGF-β RII      | N-(Mgat5)²   | Gal-3   | Membrane-endo | Cancer, stem cell | (32) |
| VEGF receptor           | N-(Mgat5)³   | Gal-3   | Membrane-endo | Neovascularization | (66) |
| Integrins               | N-(Mgat5)³   | Gal-3, -8, -9 | Focal adhesion (+) | Cancer, T cells | (34, 67, 68) |
| IL3Rβ                   | N-(Mgat5)²   | ND      | Membrane-endo | Growth control | (69) |
| N-cadherin              | N-(Mgat5)³   | Gal-3   | Cell junctions (+) | Cancer invasion | (60) |
| TRPV5, Ca²⁺ channel     | N-           | Gal-1   | Membrane     | Aging       | (70) |
| GLUT2/SLC2A2            | N-(Mgat4a)    | Gal-9   | Membrane-endo | Diabetes     | (71) |
| GLUT4/SLC2A4            | N-°          | ND      | Membrane-endo | ND          | (10) |
| B cell receptor         | N-(ST6Gal1)  | Siglec2 | Membrane, rafts, endo | B cell activation | (72) |

Gal, galactose; ND, no data.

² Sensitivity to hexosamine (UDP-GlcNAC) regulation of N-glycan branching has been tested. The gene mutation used to show lattice forming activity (29). These findings are consistent with galectin-1 forming homogeneous lattices with glycoproteins in vivo. In contrast, in vitro studies indicate that galectin-3 is a mixture of monomers and pentamers in solution, and that the latter form disorganized heterogeneous lattices with multivalent carbohydrates (30). For example, galectin-3 binds but does not selectively aggregate CD45 on the surface of apoptosis-sensitive T cells, as does galectin-1 (31). In addition, galectin-3 cross-links different cytokine receptors on the surface of cells, slowing their mobility and loss to endosomes and enhancing cellular sensitivity to ligand-dependent signaling (32, 33). The galectin-3 lattice also regulates the dynamics of receptors in a context-dependent manner. For example, galectin-3 binding reduces GFP-tagged EGF receptor mobility in the lipid bilayer while preserving sensitivity to EGF, but it increases mobility in focal adhesions and promotes PI3K signaling (33, 34) (Table I). The bi-, tri-, and tetraantennary N-glycans display increasing LacNAc epitope densities and affinity for galectins, respectively (35). However, galectin-1 and -3 differ in their tolerance of additional modification to the LacNAc epitope, which might contribute to their distinct cross-linking activities and biological properties (36).
Mgat5) each substitute the trimannosyl core at a specific
IVa/IVb, and V (encoded by Mgat1, Mgat2, Mgat4a/4b, and
Mgat5) each substitute the trimannosyl core at a specific

position in a sequential manner. GnT-IVa and GnT-IVb are
catalytically redundant and initiate the synthesis of the
GlcNAcβ1–4 branch on the core Manα1–3 arm. Loss of the
branch decreases LacNac epitopes per N-glycan and, conse-
quently, glycoprotein affinities for galectins. Mgat4a expression
is prominent in pancreatic and gastrointestinal tissues, whereas
Mgat4b is widely expressed in most tissues. Mgat4a mutant
mice develop type 2 diabetes with suppressed insulin secre-
tion by β-cells due to the aberrant N-glycans on GLUT2,
which reduce binding and surface retention by galectin-9.
Mgat4b-deficient mice show compensation in the form of a
marked up-regulation of Mgat4a expression in organs corre-
sponding to a near-normal distribution of N-glycans. As such,
the phenotype of Mgat4b-deficient mice is relatively normal,
with modest decreases in coagulation factors and prolonged
bleeding time. Although the Mgat4a/4b double deficiency
eliminated expression of the GlcNAcβ1–4 branch, increased
LacNac epitope was seen as poly-LacNAc in compensating
amounts on the remaining branches of the N-linked glycans
(37).

Mgat4a/4b double-deficient mice displayed elevated rest-
ing glucose levels, similar to the Mgat4a mice, suggesting that
compensation is insufficient. Although epitope compensation
within the same classes of glycans and glycoproteins has
been shown to maintain the residency of receptors at the cell
surface in cell culture (10), homeostasis in vivo might fail at
another level of regulation. In this regard, the mechanism of
compensation in the double null mice was an up-regulation of
multiple enzymes that act downstream of GnT-IV branching to
generate poly-LacNAc and Leα epitopes, whereas compensa-
tion in Mgat4a was an up-regulation of Mgat4b encoding
the same activity. Although these compensating enzymes
are increased in the double null mice, they have different promot-
ers and are not likely to mimic the normal epitope density and
wild-type phenotype. In this regard, the Mgat4a gene is sen-
sitive to metabolic regulation through the transcription factors
FOXA2 and HIF1A (39). However, epitope density compensa-
tion in the double null mutant mice precludes a comparison
with a truly epitope-deficient background, with which a more
severe phenotype might be expected.

Mouse Mgat4a and Mgat4b segregate independently, and
offspring from Mgat4a/Mgat4b heterozygote breeding shows
reduced survival of Mgat4b−/− pups with one or two mutant
Mgat4a alleles (37). Thus functional compensation, as mea-
sured in terms of pup survival, is less effective in double
mutant embryos than Mgat4a alone, where GlcNAcβ1–4
branching is rescued via up-regulation of Mgat4b expression.
This suggests a partially penetrant phenotype (stochastic) in
which branch-extending activities and epitope densities are
suboptimal in Mgat4a/Mgat4b mice. However, these studies
reveal systemic feedback that appears to maintain epitope
density by means of compensation on related structures,
which, in turn, should support galectin lattices.

**Fig. 2.** A, Ordinary Differential Equation (ODE) computational
model of receptor regulation by the galectin lattice. N-glycosyla-
tion site (NXS/T) multiplicity interacts with the Golgi branching path-
way to regulate glycoprotein affinities for the galectin lattice. Simula-
tions of fractional change in surface receptors are shown as a function of
the site number (n) and the UDP-GlcNAc supply to N-glycan branching (x-axis). Experimental validation of the model for EGFR (n = 8 occupied sites) and TβR/II (n = 3) can be found in Ref. 10. B, Single
epitope density and differential regulation. This is a more general
model of opposing signaling pathways with high and low site PTM
densities that allows differential regulation by the same PTM sub-
strate and modifying enzymes. C, multidimensional regulatory space.
Interacting pathways can be controlled by low affinity/specificity,
multivalent, and density-dependent PTM systems. The curves indi-
cate possible trajectories for growth signaling. Each is dependent on
the site number in protein sequences (red or blue), where interaction
with an opposing pathway (not shown) results in the specific traject-
ory. Specificity arises from low-affinity and ubiquitous PTM epitopes,
based on conditional inputs such as metabolism, stress, and develop-
mental cues.
Epitope Compensation in O-linked Glycans—Following the report by Takamatsu et al. (37), Ismail et al. (38) reported on mice deficient in core 2 β1,6-N-acetylgalactosaminyltransferase (C2GnT), a key component in the O-glycan biosynthetic pathway encoded by three genes, C2GnT1, C2GnT2, and C2GnT3. Core 2 branched O-glycans are also preferentially modified by core 4- and I-Gnt enzymes. The triple knockout mice lacked the immediate product and downstream core 4- and L-branch O-glycans. Similar to the Mga4 example above, the missing O-linked branch is observed as small quantities of LacNAc epitope on the remaining linear arm of the O-linked glycans. This compensation was observed in the gastrointestinal tract but not in the kidneys, unlike in the GnT-Iva/Vb double-deficient mice, where N-glycan compensation was observed in all organs examined, including the kidneys. The apparent absence of O-glycan epitope compensation in the kidneys indicates that O-glycan compensation in the gastrointestinal tract and, by extension, N-glycan compensation are active processes that are not due to random biosynthetic processing. Surprisingly, O-linked mannosyl glycans were up-regulated in the triple knockout mice. This is an independent pathway that generates O-Man-GlcNAc-galactose-sialic acid at different sites on proteoglycans in the CNS independent pathway that generates O-Man-GlcNAc-galactose-sialic acid at different sites on proteoglycans in the CNS.

Metabolic Regulation of Epitope Density on Receptors—Gastric lattice formation at the cell surface is highly dependent on N-glycan branching and the LacNAc epitope density on transmembrane glycoproteins (48). Therefore, the expression and biochemical properties of the Golgi enzyme have a considerable effect on the epitope density. Many of the Golgi enzymes function at subsaturating concentrations of a substrate, either the acceptor N-glycans on glycoproteins or donor sugar-nucleotide ($K_m$ values in Fig. 1). This results in a heterogeneous distribution of LacNAc epitopes at the various NXS/T sites, consistent with a model dependent on epitope density, rather than targeted occupancy at specific sites in glycopolypeptides. We developed a computational model of epitope-density-dependent regulation of receptor residency at the cell surface via binding to galectin-3 (10). The Golgi output of remodeled N-glycans was computed as a function of increasing hexosamine pathway activity (i.e. UDP-GlcNAc concentrations), and the probabilistic glycoform distributions were computed for EGF receptors (EGFR) and TGF-β receptors (TβR), and then surface receptor levels due to association with the lattice and the capacity for ligand-dependent signaling (10). TβRI/II has only three N-glycans and is therefore more dependent on the UDP-GlcNAc supply and branching to generate affinity for galectin-3 than EGFR, with eight N-glycans (Fig. 2A). In other words, more epitope per glycan is required for TβRI/II, whereas with EGFR, a similar affinity for galectin-3 can be attained with more N-glycans (NXS/T sites) and less branching. Epitope equivalence or compensation is a critical feature of receptor regulation by the lattice (Table I).

Experimental data from cell lines and human autoimmune disease support the lattice model in which the N-glycan number and the Golgi pathways co-regulate receptor titration into the galectin lattice (10) (Fig. 2C). These intriguing dynamics are dependent on a conserved biochemical feature of the N-glycan branching pathway, namely, multi-step ultrasensitivity to UDP-GlcNAc. Multi-step ultrasensitivity arises from the decreasing affinities of Mga1, -2, -4, and -5 enzymes for UDP-GlcNAc in sequential order of their action. Mass spectrometry analysis indicates that bi-, tri-, and tetraantennary N-glycans increase with intracellular UDP-GlcNAc concentrations (10). In Mga5-deficient cells, the LacNAc density is
reduced but can be restored via GlcNAc supplementation to UDP-GlcNAc, which generates more bi- and triantennary glycans in the absence of tetraantennary (Mga5t) structures. Titration of UDP-GlcNAc fully restores the ordered association of EGFR and TjβR into the galectin-3 lattice, growth/proliferation, and then feedback inhibition via TGF-β/Smad signaling. A single stimulus, UDP-GlcNAc, promotes a Michaelis–Menten and a sigmoidal response for high and low multiplicity receptors, respectively, and the intervening “delay” allows growth signaling to prevail before the onset of negative regulation by the low multiplicity receptors (Fig. 2B). Consistent with this model, growth receptor kinases display roughly five times as many N-glycosylation sites (NXS/T), higher site densities, and longer extracellular domains than receptors that mediate differentiation and arrest (10). The evolution rate of glycoproteins is accelerated relative to that of proteins inside the cell (49). Moreover, the evolution of NXS/T multiplicity in receptors suggests that the delay in opposing signaling pathways has increased in humans since a common ancestor with mice.

The T cell co-receptors CD28 (n = 5 sites in humans, 4 in mice) and CTLA-4 (n = 2 in humans, 3 in mice) show a similar order of titration into the lattice in response to UDP-GlcNAc. UDP-GlcNAc levels, N-glycan branching, and poly-LacNAc increase with T cell activation (50, 51). CD28 stimulates growth, and as UDP-GlcNAc concentrations increase, surface CTLA-4 is recruited to the lattice, reaching a critical level that suppresses T cell proliferation (10, 52). A human polymorphism in CTLA-4 (49A/G, rs231775) (53) reduces N-glycan occupancy at one of the two NXS/T sites and increases the risk of autoimmune disease (54). A hyperactive variant of MGAT1 (IVsV试着T) suppresses branching, consistent with the ultrasensitive model of the branching pathway described above (52). Co-inheritance of MGAT1 (IVsV试着T) and CTLA-4 (49A/G, rs231775) additively weaken the affinity of CTLA-4 for the lattice and increase the risk of autoimmune disease (multiple sclerosis) (52). The effect of these alleles on T cell hyper-sensitivity is reversed by supplementation with GlcNAc, which is converted to UDP-GlcNAc and increases N-glycan branching. The MGAT5 (rs3814022, rs4953911) allele (55) has also been linked to multiple sclerosis severity. Moreover, IL2RA*T (rs2104286) and IL7RA*C (rs6897932) variants also drive T cell autoimmunity through N-glycan branching (52). Vitamin D3 deficiency, another well-documented risk factor, suppresses branching and T cell activation by up-regulating the expression of MGAT1. Moreover, oral GlcNAc treatment prevents spontaneous autoimmune diabetes (56) and inhibits experimental autoimmune encephalomyelitis in mice when treatment is initiated after disease onset (57). In the latter study, oral GlcNAc increased N-glycan branching and suppressed disease by inhibiting Th1 and Th17 T-helper cell responses. The “hexosamine branching lattice” provides a conceptual basis for the regulation of glycoprotein dynamics at the cell surface based on epitope density and allows for mechanisms of compensation and remarkable plasticity (11, 58) (see Figs. 2A–2C and Table I).

CONCLUSIONS

To summarize, glycan epitope compensation has been observed in mouse tissues for N- and O-linked glycans and brain gangliosides, which serves as strong evidence of systemic feedback. Density can be maintained through the gene expression of biosynthetic enzymes of the Golgi and the supply of metabolites to the hexosamine pathways. Changes in the lectin–glycan lattice-dependent regulation of receptors can act as an environmental sensor and result in altered signaling to metabolism and Golgi enzyme expression and thus epitope compensation. Importantly, different arrangements of epitopes in N-glycans produce distinct glycoform distributions but maintain comparable affinities for galectins. Therefore, epitope equivalence might allow the cell surface to adapt to various environmental inputs and stresses. Adaptation might involve the shifting of epitopes between different glycan classes. For example, gangliosides are concentrated in lipid rafts (59, 60) and might help recruit glycoproteins into lattices in rafts or, alternatively, compete for galectin that would otherwise bind N-glycans on receptors outside of rafts.

The ultrasensitive response of the branching pathway to UDP-GlcNAc, and by extension metabolism, is embedded in the kinetics and gene expression properties of MGAT enzymes (10, 11). More generally, ultrasensitive responses provide a means of decisive all-or-nothing transitions in the cell cycle and development, where multivalency often plays a role as well. A small shift in PTM epitope density can promote decisive transitions in molecular complex formation and signaling (61). These effects are present in other classes of PTMs, such as phosphorylation (61). For example, the S. cerevisiae cyclin dependent kinase (CDK) inhibitor Sic1 has nine sites in unstructured regions of the protein that are progressively phosphorylated as the G1 phase progresses (21, 22). Six phosphorylated sites are required for a threshold level of Ccd4 binding to Sic1, which triggers its ubiquitination and proteolysis, thereby relieving the inhibition of CDK and triggering the G1/S transition in a switch-like or ultrasensitive response. This ensures an all-or-nothing decisive start to DNA replication. Ultrasensitive responses are seen widely in regulatory systems, and many depend on affinity enhancement via conditional regulation through PTMs. Phosphorylated sites do not undergo secondary modification of the phospho-amino acids, but ubiquitinated sites can become polyubiquitin, generating multiple epitopes for ubiquitin-binding proteins, analogous to LacNAc units in branched N-glycans. For example, anaphase-promoting complex ubiquitinates substrates that are modified and degraded in a specific sequence that orders cell cycle events. Anaphase-promoting complex catalyzes the polyubiquitination of substrates with different relative processesivity (62). Processive substrates obtain many ubiquitin chains within a single anaphase-promoting complex binding event,
whereas distributive substrates frequently dissociate between substitutions (63). In a manner similar to that of N-glycan epitopes in the regulation of receptors, the differential modification of proteins by a common donor can time events in the cell cycle. Lastly, PTMs such as phosphate, methyl, and acetyl groups on peptides and glycan epitopes generally present modest affinity to binding partners (17). Therefore, biological responses to the modification of PTM sites might depend on epitope densities and multivalent binding interactions (11). The encoded number of PTM sites in proteins, and their non- or partially ordered occupancy, often generates a characteristic ultrasensitive response to biologically important cues (Fig. 2B). Therefore, specificity arises from low-affinity and multiple PTM epitopes in target proteins and the threshold number of modifications required in order to reach the epitope density for binding partners. Many other factors affect the target proteins differentially, such as the modifying enzymes and substrate levels for modification and, ultimately, the homeostatic opposing pathways (Fig. 2C).

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