The Early Metazoan *Trichoplax adhaerens* Possesses a Functional O-GlcNAc System*

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Background: Protein O-GlcNAcylation and orthologues of O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) occur separately or together in all kingdoms of life.

**Results:** The basal metazoan *Trichoplax adhaerens* is the simplest organism to possess functional OGT, OGA, and protein O-GlcNAcylation together.

Conclusion: Reversible protein O-GlcNAcylation is conserved throughout the metazoan lineage.

Significance: *T. adhaerens* can be used as a reductionist model to identify evolutionarily conserved O-GlcNAc targets.

O-GlcNAcylation is a reversible post-translational signaling modification of nucleocytoplasmic proteins that is essential for embryonic development in bilateria. In a search for a reductionist model to study O-GlcNAc signaling, we discovered the presence of functional O-GlcNAc transferase (OGT), O-GlcNAcase (OGA), and nucleocytoplasmic protein O-GlcNAcylation in the most basal extant animal, the placozoan *Trichoplax adhaerens*. We show via enzymatic characterization of *Trichoplax* OGT/OGA and genetic rescue experiments in *Drosophila melanogaster* that these proteins possess activities/functions similar to their bilaterian counterparts. The acquisition of O-GlcNAc signaling by metazoa may have facilitated the rapid and complex signaling mechanisms required for the evolution of multicellular organisms.

Post-translational protein O-GlcNAcylation is the reversible addition of β-D-N-acetylglucosamine (GlcNAc) to serine and threonine residues on metazoan nucleocytoplasmic proteins (1). O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) are the enzymes responsible for the addition and removal of O-GlcNAc, respectively. Since it was first described in 1984 (2), O-GlcNAc has become associated with a range of cellular processes (1). There appears to be extensive cross-talk between O-GlcNAc and Ser/Thr phosphorylation, with the two modifications occurring at the same or neighboring residues on proteins (3–5). O-GlcNAcylation of several kinases (AMP-activated protein kinase, CaMKIV, and CaMKII, for example) regulates their activity, and OGT functionally interacts with two catalytic subunits of protein phosphatase 1 (6–9). The discovery of O-GlcNAc on proteasome subunits in *Drosophila* implicates a role for this post-translational modification in protein trafficking and degradation (10). O-GlcNAcylation increases in the presence of stressors like heat and heavy metals (11, 12), and protects cardiac tissues following ischemia (13). Reports have emerged of the involvement of O-GlcNAc in gene expression and epigenetics. The discovery of O-GlcNAc on RNA polymerase II transcription factors suggested a role for OGT in mitotic cells was shown to alter methylation and phosphorylation of histone H3 (15). In addition, cell-cycle-dependent O-GlcNAc cycling was also found to occur on histones H2A, H2B, and H4 (16). Transcriptional repression by OGT involving interactions with mSin3A and HDAC1 has been demonstrated (17). Activation of gene expression downstream of H2B O-GlcNAcylation has been characterized, as well as transcriptional changes due to H3K4 trimethylation facilitated by the TET protein-OGT complex (18, 19). O-GlcNAcylation is associated with disease conditions like Type II diabetes, Alzheimer disease, and cancer (1, 20).

Following the identification of OGT and OGA activities (21–23) and their enzymatic characterization (23, 24), transcripts have been cloned from humans and other organisms (25, 26) and found to be highly conserved in animals. In humans, a single *OGT* gene encodes three isofoms of the protein, the longest, nucleocytoplasmic OGT (ncOGT/hOGT), is a ~116 kDa protein and possesses 13.5 tetratricopeptide repeats (TPRs) at its N terminus (27, 28). Another isofrom possessing 9.5 TPRs and a mitochondrial localization signal (mOGT ~103 kDa) is targeted to mitochondria. The shortest OGT isofrom (sOGT ~78 kDa) contains only 2.5 TPRs and also has nucleocytoplasmic localization (27). In *Drosophila* and *Caenorhabditis elegans*, single *ogt* genes encode a single protein similar to...
human ncOGT (27, 29–31). Zebrafish is exceptional among animals to possess two ogt genes encoding six variants of the protein at different stages of development (32).

In humans, a single gene encodes two isoforms of OGA. The longer cytoplasmic isoform (hOGA ~ 130 kDa) possesses an N-terminal catalytic domain and a C-terminal histone acetyltransferase (HAT)-like domain, whereas the shorter nuclear and lipid-droplet targeted isoform (~75 kDa) lacks the HAT-like domain (33, 34). In C. elegans, a single oga gene encodes four major transcripts generated by alternative splicing and intron utilization to produce proteins of different lengths containing both the catalytic and HAT-like domains (35). Drosophila has a single oga gene encoding a single protein. Toleman et al. (36) demonstrated HAT activity for hOGA purified from mammalian cells, which was, however, not observed in a subsequent study (37). Structural characterizations of putative bacterial acetyltransferases sharing sequence conservation with the HAT-like domain of hOGA enforce that hOGA lacks HAT activity (38, 39). Furthermore, the bacterially expressed hOGA HAT-like domain does not bind acetyl-CoA in vitro (38).

Although strides have been made toward identifying the processes regulated by O-GlcNAcylation, uncovering the consequences of O-GlcNAc on individual proteins at an organismal level remains a challenge. Gene knock-out is a useful strategy to addressing the challenge by generating animals lacking OGT/OGA activity. However, the fact that Ogt null mice and Dro sophila ogt mutants die at different stages of development and Oga null mice as neonates (30, 40, 41) limits their use for functional studies. Whereas levels of OGT and OGA have been manipulated in zebrafish embryos and Xenopus laevis oocytes to study the roles of O-GlcNAc in development (42, 43), knock-outs of the enzymes have not been reported in these organisms. C. elegans is the only known example of an organism that remains viable and fertile after loss of OGT and OGA activity (29, 35). ogt and oga null mutants of C. elegans have therefore been used to study the effects of O-GlcNAc cycling on lifespan and aging (44–46). Accessible reductionist models with smaller O-GlcNAc proteomes are thus invaluable toward accelerating research into understanding the conserved roles and mechanisms of protein O-GlcNAcylation. The aim of this study was to find another such model.

Here, we report that the basal metazoan Trichoplax adhaerens is the simplest organism to possesses both OGT and OGA and O-GlcNAcylated proteins. OGT appears to be expressed throughout the body of Trichoplax under basal conditions. Trichoplax OGT can rescue pupal lethality of the Drosophila sxc (ogt) mutant in addition to compensating for the maternal requirement of OGT. Trichoplax OGA can de-O-GlcNAcylate human and Drosophila cell lysates. Together, these data imply that the acquisition of OGA by metazoans at the time of diverging from their unicellular ancestors facilitated the cycling of O-GlcNAc on proteins. This acquisition may have expanded the repertoire of complex signaling mechanisms required for metazoan-specific features absent in other intracellular OGT-possessing organisms lacking OGA.

**EXPERIMENTAL PROCEDURES**

**Sequences and Alignments**—Orthologues of OGA and OGT in Trichoplax were identified by using BLAST in the Uniprot database and the Trichoplax genome database. Query sequences were from the following: Homo sapiens, Mus musculus, Danio rerio, Drosophila melanogaster, and C. elegans. Sequences were aligned using CLUSTALW, and edited and annotated with ALINE. XtalPred and sequence alignments with OgOGA were used to predict regions of structural disorder in hOGA, DmAOGA and the TaOGAs. Surface views of hOGT and OgOGA were generated and colored by similarity to their Trichoplax counterparts using PyMOL.

**T. adhaerens Culture and Harvest**—Starter cultures of T. adhaerens and the cryptomonad marine red alga Rhodomonas salina, which serves as a food source for Trichoplax, were obtained from Prof. Leo Buss (Yale University). Trichoplax were seeded and grown on a mat of monoculture of Rhodomonas in 150-mm glass Petri dishes at 22 °C in artificial seawater (Reef Crystals, Aquarium Systems) of 36 parts per thousand (4.5 brix %) salinity supplemented with 0.1% (v/v) Micro Algae Grow (Florida Aqua Farms). To harvest Trichoplax, culture medium in Petri dishes was gently pipetted up and down several times to lift adherent animals off the glass surface. The contents of the dish were then centrifuged at 1000 × g at 4 °C for 10 min. The algae were removed by washing with unsupplemented artificial seawater by repeated centrifugation at low speed.

**Rapid Amplification of cDNA Ends (RACE)**—Trichoplax total RNA was extracted using TRI reagent (Sigma). cDNA was synthesized using Precision qScript™ Reverse Transcription kit (Primer Design) and an oligo(dT) primer or the First-Choice® RLM-RACE Kit (Ambion). Full-length coding sequences for Trichoplax OGA and OGT were determined using the FirstChoice® RLM-RACE Kit (Ambion) according to the manufacturer’s instructions. PCR products were gel purified and sequenced. Full-length sequences were then amplified from cDNA and cloned into pCR®-Blunt II-TOPO® (Invitrogen) for sequence verification. Two to four colonies were sequenced using both the M13-F and M13-R primers.

**Cloning and Site-directed Mutagenesis**—TaOGA53 and TaOGA54 were cloned into pGEX6P1 and pOPTH, respectively, using a previously described restriction-free method (47) from TOPO clones after RACE experiments identified start and end sites. N-terminally truncated TaOGT was initially cloned through PCR amplification followed by BamHI-Sall digestion and ligation into pGEX6P1. Following identification of the start of TaOGT through RACE experiments, a missing segment was added to the existing construct by the restriction free cloning method. Site-directed mutations were introduced using the Stratagene QuikChange Site-directed mutagenesis kit except KOD Polymerase (Novagen) was used instead of Pfu, and DpnI was purchased from Fermentas. The presence of the intended mutations was confirmed by DNA sequencing.

**Protein Expression and Purification**—Plasmids containing TaOGT and TaOGA53 were transformed into Escherichia coli ArcticExpress competent cells (Stratagene), whereas TaOGA54 and hCK2α were transformed into E. coli BL21(DE3) pLysS cells. Cells were grown overnight at 37 °C in
Luria-Bertani medium containing 50 μg/ml of ampicillin (LB-Amp) and used at 10 ml/liter to inoculate 6 liters of fresh LB-Amp in the case of BL21(DE3) pLysS cells and 12 liters for ArcticExpress cells. BL21(DE3) pLysS cells were grown to an A600 of 0.6–0.8, transferred to 18 °C, and induced with 250 μM isopropyl 1-thio-β-d-galactopyranoside and harvested after 16 h. ArcticExpress cells were grown to an A600 = 1.0, transferred to 12 °C, and induced with 250 μM isopropyl 1-thio-β-d-galactopyranoside and harvested after 72 h by centrifugation for 30 min at 3500 rpm (4 °C). Cell pellets were resuspended in 10–20 ml/liter of 50 mM Tris, 250 mM NaCl, and 0.5 mM Tris(2-carboxyethyl)phosphine (lysis buffer) at pH 9.0 for TaOGT and hCK2α and pH 7.5 for TaOGA53 and TaOGA54. Lysis buffers for TaOGT and TaOGA53 also contained 5% glycerol and 0.05% Nonidet P-40. All lysis buffers were supplemented with protease inhibitors (1 mM benzamidine, 0.2 mM PMSE, and 5 μM leupeptin), DNase and lysozyme prior to lysis. Cells were lysed using a continuous flow cell disruptor (Avestin, 3 passes at 20 kpsi) and the lysate was cleared by centrifugation (30 min, 15,000 rpm, 4 °C). Supernatants were collected and loaded onto 2 ml of glutathione-Sepharose (GE Healthcare Life Sciences) pre-equilibrated with lysis buffer. TaOGA54 was loaded on to 2 ml of IMAC Sepharose (GE Healthcare) charged with NiSO4 and pre-equilibrated with lysis buffer. Loaded resins were each washed with 500 ml of lysis buffer or lysis buffer containing 30 mM imidazole in the case of IMAC resin. The ArcticExpress chaperones were removed from captured GST-tagged proteins by washing the resin with 1× TBS (25 mM Tris, pH 7.5, 150 mM NaCl) containing 10 mM ATP and 11 mM MgCl2 (4× washes at 37 °C). GST-tagged proteins were eluted from resin by cleavage of the GST tag using GST-tagged PreScission™ protease at 4 °C for 16 h. His6-tagged TaOGA54 was eluted using lysis buffer containing 250 mM imidazole and dialyzed into 1× TBS containing 0.5 mM Tris(2-carboxyethyl)phosphine. TaOGA54 and hCK2α were further purified by size exclusion chromatography using a Superdex 200, 26/60 column. All proteins were concentrated using spin concentrators and purity was assessed by SDS-PAGE followed by Coomassie R-250 staining. Point mutants of TaOGT, TaOGA53, and TaOGA54 were purified the same way as their wild type counterparts.

**Steady-state Kinetics—\( K_{\text{m}} \) for UDP-GlcNAc of wild type and mutant TaOGT was determined as described previously (48). Briefly, 100 μl reactions contained 100 nM TaOGT in 50 mM Tris, pH 7.5, 0.1 mg/ml of BSA, 10 μM sodium dithionite, and 100 μM peptide (KKNESPATVPVSTA) and varying amounts of UDP-GlcNAc. Reactions were carried out for 75 min at room temperature and stopped using 200 μl of 37.5 μM fluorophore (48–50) prepared in 50 mM HEPES, pH 7.5, 10 mM NaCl, and 50% (v/v) methanol. Fluorescence was measured using Gemini EM plate reader (Molecular Devices) with excitation and emission wavelengths of 485 and 530 nm, respectively. The IC50 for Goblin 1 was determined using 13 μM UDP-GlcNAc, 100 μM peptide, and varying concentrations of the inhibitor. Steady-state kinetics of wild type and mutant TaOGA54 and TaOGA53 were determined as described (51) using 4-methylumbelliferyl-N-acetyl-β-d-glucosamine (4MU-NAG, Sigma). Reaction mixtures (100 μl) contained 2–100 nM enzyme in 1× TBS, 0.1 mg/ml of BSA, and varying amounts of substrate in 1–2% dimethyl sulfoxide. Reactions were performed for 30–120 min at room temperature and stopped by the addition of 200 μl of glycine-NaOH, pH 10.3. Fluorescence of released 4-methylumbellifereone was measured using a Synergy 2 plate reader (Bio-Tek), with excitation and emission wavelengths of 360 and 460 nm, respectively. IC50 values were measured at \( K_{\text{m}} \) with varying concentrations of inhibitors. All experiments were performed in triplicate and measurements were corrected for background emission from reactions containing no peptide (for OGT assays) or no enzyme (for OGA assays). For all assays performed, substrate turnover was under 10%. Non-linear regression curves were fitted with Prism (GraphPad).

**In Vitro O-GlcNAcylation of hCK2α—**Reactions contained 0.25 μg of hCK2α, 3.7 mM UDP-GlcNAc, and 2.5 μM of either hOGT/GST-hOGT (purified as described previously (52)) or TaOGT in a total volume of 10 μl of 10 mM Tris, pH 7.5, and 1 mM DTT and incubated at room temperature for 1.5 h. For subsequent TaOGA treatments, GST-hOGT was pulsed out of the reactions using glutathione-Sepharose and residual hOGT activity was blocked using 5 mM UDP. Reactions were stopped by the addition of Laemmli buffer and proteins were separated by SDS-PAGE and analyzed by Western blotting as described below.

**Drosophila Genetics and Adult Fly Lysates—**The following stocks were used: w1118, sxe1/Cyo, sxe9/Cyo, and tub::GAL4/TM6. Transgenic flies were generated by Rainbow Transgenic Flies Inc., CA, with the attP insertion site at 86F8. 5 anesthetized male adult flies were frozen on dry ice and homogenized in 50 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 μM GlcNAcstatin C, 5 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM benzamidine, 0.2 mM PMSE, 5 μM leupeptin, and 1 mM DTT), following which an equal volume of 3× SDS Laemmli buffer was added. Lysates were then boiled for 5 min at 95 °C, centrifuged at 16,000 × g for 10 min, and supernatants were collected. 30 μg of crude lysates were used for Western blots.

**Cell Culture, Lysis, and Protein Extraction—**HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Gibco) and antibiotics (Gibco) at 37 °C in a humidified atmosphere. Drosophila S2 cells were cultured in Schneider’s medium supplemented 1-glutamine, 10% FBS (Gibco), and antibiotics (Gibco) at 25 °C. HEK293, S2 cells, and Trichoplax were lysed in 10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 μM GlcNAcstatin C, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and protease inhibitors. Lysates were cleared by centrifugation. Bradford assay or a Pierce 660-nm protein assay was used to quantify cell lysates.

**Transfections, RNAi, and Enzymatic Treatments of Lysates—**S2 cell transfections were carried out by mixing FuGENE HD (Roche), DNA (2 μg) at a 3:2 ratio (μl/μg) in 100 μl of sterile water. The constructs used for transfections were pMT-GAL4, pUAS-DmOGTWT-HA, pUAS-TaOGTWT-HA, and pUAS-TaOGTβ155M-HA. The metallothionein promoter was induced with 1 mM CuSO4 24 h after transfection. RNAi was performed
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48 h before DNA transfections by transfecting 4 μg of double-stranded RNA directed against the 3‘ UTR of DmOGT transcript. Double-stranded RNA was synthesized using a TranscriptAid T7 High Yield Transcription Kit (Thermo) according to the manufacturer’s instructions from PCR products containing T7 (indicated in lowercase in the primer sequences) sites introduced by the following primers: forward, taatagctactatataggCCATTTTTGGTGTTT, antisense reverse, taatagctactatataggTTCCATTGTTT, antisense forward, CGCATGAAATCTTTGTTT, sense reverse, TCTGCGTATTATATCTGATA.

20 μg of lysates were subjected to all enzymatic treatments. PNGase F (New England Biolabs) treatment was performed as described by the manufacturer. CpNagl (purified as described in Ref. 51, but with the GST tag left uncleaved at the N terminus) treatment was performed with 2–4 μg of the enzyme at 37 °C for 1 h. TaOGA54 and TaOGA53 treatments were performed on lysates and in vitro O-GlcNAcylated hCK2α with 5, 10, or 15 μg of the enzymes for 4 h at room temperature. Labeling of lysates with GalT1 (Y289L) was performed according to the manufacturer’s instructions (Invitrogen).

Western Blotting—Proteins were resolved in SDS-PAGE gels and blotted onto nitrocellulose or PVDF membranes. Polyclonal antibodies were generated by immunizing rabbits (Dundee Cell Products) with a pair of peptides from each protein (TaOGT, EYADHYSEKLAFLPNS and TRLRKLQDKI; TaOGA, taatacgactcactatagggCGC-

RESULTS

Trichoplax Expresses Orthologues of Metazoan OGT and OGA—We aimed to identify basal organisms possessing both OGT and OGA genes in an attempt to identify a reductionist model to probe O-GlcNAc signaling and shed light on the evolution of reversible intracellular protein O-GlcNAcylation. Reports have suggested the presence of O-GlcNAcylated proteins in filamentous fungi (54), protists (55, 56), and bacteria (57, 58). Plants and primitive eukaryotes possessing apparent OGT orthologues and O-GlcNAcylated proteins appear to lack OGA (55, 56, 59), suggesting that O-GlcNAcylation is either irreversible in these organisms, or may be reversed by unidentified enzymes bearing no similarity to metazoan OGA. Conversely, in the bacteria in which O-GlcNAc has been found, bioinformatics searches did not identify OGT-like proteins. We parsed the CAZy database, which lists a number of other organisms ranging from archaea to man that possess enzymes of the glycosyltransferase family 41 (GT41) and the glycoside hydrolase family 84 (GH84) to which OGT and OGA, respectively, belong. Upon close examination, it is clear that of all these organisms, only metazoa possess clear orthologues of both OGT and OGA (bearing over 40% sequence identity to hOGT and hOGA) in their genomes. We then searched the genomes of basal metazoa and identified an OGT gene fragment and two
candidate OGA gene fragments in the recently sequenced genome of T. adhaerens (60), the sole member of phylum placoza. Trichoplax is a free-living marine organism considered to be one of the most basal extant multicellular organisms existing at the boundary between unicellular eukaryotes and metazoas (60–62). It contains only six cell types organized in three cellular layers (61, 63). The presence of putative stem cells at the periphery of the body of Trichoplax has been hypothesized (53), but remains unconfirmed (63).

Upon identifying fragments of ogt and oga genes in Trichoplax using bioinformatics, we performed 5' and 3' RACE (rapid amplification of cDNA ends) to obtain full-length sequences of these genes. It emerged that Trichoplax OGT (TaOGT), apart from having the catalytic domain, contains 13.5 N-terminal TPR repeats and a putative bipartite nuclear localization signal like hOGT (64) (Fig. 1a, supplemental Fig. S1a). It shares 66 and 64% overall amino acid sequence identity with hOGT and D. melanogaster OGT (DmOGT), respectively (Fig. 1a, supplemental Fig. S1a). Its active site is conserved with that of hOGT (supplemental Fig. S1b), and contains the key lysine residue (Lys-815 in TaOGT and Lys-842 in hOGT) (Fig. 1a, supplemental Fig. S1a), shown to be critical for the activity of hOGT (52). The most variable region is the intervening domain within the catalytic lobes of the enzyme, whereas the TPRs are the most conserved (supplemental Fig. S1a). Unlike other metazoans, which possess a single oga gene, the genome of Trichoplax encodes for two putative OGAs, TaOGA53 and TaOGA54 (after their Uniprot IDs B3SB53 and B3SB54). TaOGA53 resembles the shorter hOGA isoform lacking the HAT-like domain, whereas TaOGA54 is similar to the full-length hOGA (Fig. 1b, supplemental Fig. S2a). The glycoside hydrolase domains of TaOGA53 and TaOGA54 share 60% sequence identity with each other and are over 50% identical in amino acid composition to the glycoside hydrolase domain of hOGA and D. melanogaster OGA (DmOGA). The Asp-Asp motif shown to be important for hOGA activity (65) is conserved in the TaOGAs (Fig. 1b, supplemental Fig. S2a). The TaOGAs are also both about 40% identical to the structurally characterized bacterial OGA from Oceanicola granulosus (OgOGA) (66), with which they share a conserved active site (Fig. 1b, supplemental Fig. S2, b and c). Neither TaOGA53 nor TaOGA54 appears to have a caspase 3-cleavage site, a feature present in hOGA (Fig. 1a, supplemental Fig. S2a) (37).

For immunodetection of TaOGT and the TaOGAs, antibodies were raised against two unique peptides within each protein. However, antigen-purified antibodies could only detect their respective recombinant proteins and not endogenous TaOGT or TaOGA53 and TaOGA54 in lysates by Western blotting or immunoprecipitation. This was probably either due to low expression levels of these proteins or weak affinity of the antibodies toward them. Nevertheless, analysis of published high-resolution proteomics reveals the presence of TaOGT and TaOGA54 in the Trichoplax proteome when the organism is cultured under standard culture conditions (67).

Reports have suggested the existence of a ring of putative stem cells at the periphery of the body of Trichoplax (53), where specific orthologues of the developmental genes Hox, T-box, and Pax have been shown to be expressed (53, 67–70). Given that Ogt is part of the polycomb group of developmental genes in Drosophila (30) and has been shown to be essential for stem cell viability in mice (40), we wanted to investigate localization of OGT in Trichoplax to explore its potential function in this basal metazoan. In the absence of a robust antibody for immunofluorescence staining, we performed in situ hybridization to localize OGT transcripts in Trichoplax whole mounts. Dioxygenin-labeled antisense probes were used and sense controls were performed in parallel. To detect hybridized probes, we used alkaline phosphatase-conjugated anti-dioxigenin antibodies and a substrate that turns purple upon reacting with alkaline phosphatase. In contrast to the transcripts of the aforementioned developmental genes, OGT transcripts were not restricted to the periphery of the organism. Instead, we observed that OGT transcripts were distributed evenly in the organism in samples probed with antisense RNA (n = 12) with negligible staining in those probed with the sense control (n = 12) (Fig. 1c). This pattern is similar to the expression of the ubiquitous actin (53) and suggests that OGT, which is expressed in several tissues in higher organisms (71), may also be expressed and have functions in the different cell types of a basal organism like Trichoplax.

TaOGT Is a Functional O-GlcNAc Transferase—We cloned, recombinantly expressed, and purified the putative TaOGT to investigate its activity and elucidate its biochemical properties. The negative control for this experiment was the Lys-815 (TaOGTK815M) mutated to Met because the equivalent Lys-842 residue in hOGT is indispensable for catalysis (52). Steady-state kinetics were performed employing a recently published fluorescence assay (48). The K_m for UDP-GlcNAc was measured in the presence of excess peptide substrate (KKENPVSTA, previously used as a substrate to measure the activity of hOGT (48)) and was found to be 13 ± 2 μM, within the range reported for hOGT (24, 72–74) (Fig. 2a). TaOGT activity is inhibited by the OGT bisubstrate inhibitor Goblin 1 (48) with an IC_{50} (27 μM) comparable with that reported for hOGT (Fig. 2b). Furthermore, TaOGT_{WT}, but not the inactive mutant TaOGT_{K815M}, could O-GlcNAc modify human CK2α (hCK2α), a well characterized hOGT substrate (75–77) in vitro, thus validating it as a true OGT orthologue (Fig. 2c). This experiment also revealed that TaOGT_{WT}, like its full-length human counterpart (75), undergoes autoglycosylation as evidenced by the reactivity of the anti-O-GlcNAc antibody RL-2 toward TaOGT_{WT} but not TaOGT_{K815M} (Fig. 2c).

TaOGT Rescues Drosophila supersex combs (sxc) Lethality—Drosophila OGT mutants, also known as supersex combs (sxc) mutants, die as pharate adults and this lethality can be rescued by ubiquitous expression of transgenic wild type DmOGT in sxc transheterozygotes (31). We used this approach to investigate the functional equivalence of TaOGT and DmOGT. Initial experiments were performed in S2 cells where endogenous OGT was knocked down using RNAi directed towards the 3' UTR of DmOGT and cells were transfected with plasmids carrying either TaOGT_{WT} or the catalytically inactive TaOGT_{K815M}. O-GlcNAc levels in cells transfected with TaOGT_{WT}, but not TaOGT_{K815M}, were restored to levels comparable with cells transfected with DmOGT (Fig. 3a). Strikingly, in the context of the whole organism, the number of sxc
transheterozygotes recovered on rescue with TaOGT (13% of total progeny) was comparable with that of the DmOGT (26% of total progeny). The level of rescue with DmOGT is twice as that of TaOGTWT because the transgenic line used in the case of DmOGT was homozygous (Fig. 3b, Table 1). In the control crosses lacking either the driver or the transgene, no adult sxc1/sxc6 transheterozygotes were recovered. sxc is a maternal effect gene and the rescue by TaOGT to produce F1 progeny is the rescue of the zygotic requirement of OGT. To assess whether the maternal OGT function could also be rescued by TaOGT, the rescued F1 males were crossed with rescued F1 females. The only completely functional OGT in this cross is the TaOGTWT.

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**Diagram a:**
- hOGT
- DmOGT
- TaOGT

**Diagram b:**
- hOGA
- DmOGA
- TaOGA53
- TaOGA54
- OgOGA

**Diagram c:**
- Antisense
- Sense
  - 10 X
  - 40 X
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FIGURE 1. *Trichoplax* possesses OGT and OGA orthologues. a, schematic showing the domains of TaOGT compared with hOGT and DmOGT. The TPR region of the proteins is shown in green, the N- and C-terminal catalytic lobes (GT41-N and GT41-C) in purple, and the intervening domain (Int-D) in peach. The conserved lysine residue required for catalytic activity of the OGTs is shown, as is the conserved nuclear localization signal (NLS) in the proteins. b, schematic showing the domain architecture of the TaOGAs compared with hOGA, DmOGA, and OgOGA (a structurally characterized bacterial OGA with high sequence conservation with hOGA (66)). The catalytic domain with glycoside hydrolase activity (GH84) is shown in pale salmon, c, localization of TaOGT transcripts analyzed by whole mount in situ hybridization using dioxygenin-labeled probes. Hybridized probes detected using alkaline phosphatase (AP)-conjugated anti-dioxygenin antibody and the AP substrate BM purple. Purple staining throughout the organism shows ubiquitous presence of TaOGT transcripts. Scale bars: top panels, 100 μm; bottom panels, 20 μm.

FIGURE 2. TaOGT is a functional O-GlcNAc transferase. a, Michaelis-Menten kinetics of TaOGT measured using the peptide substrate KKENSPAVTPVSTA and varying amounts of UDP-GlcNAc. Reactions were carried out for 75 min at room temperature and read after the addition of a compound that fluoresces upon binding to the reaction product UDP (48–50). Data points were fitted to the Michaelis-Menten equation using Prism (GraphPad). Experiments were performed in triplicate and error bars represent mean ± S.E. h, IC₅₀, was measured using UDP-GlcNAc at a concentration equal to the Kₘ. Highest activity in the absence of inhibitors is arbitrarily set as 100%. Data points were fitted to a three-parameter equation for dose-dependent inhibition using Prism (GraphPad). Experiments were performed in triplicate and error bars represent mean ± S.E. c, in vitro O-GlcNAcylation of hCK2α by TaOGT and autoglycosylation of TaOGT detected by Western blotting using the anti-O-GlcNAc antibody RL-2. hCK2α was incubated with TaOGT and OGA54 and a molar excess of UDP-GlcNAc at room temperature for 1.5 h. Negative controls include hCK2α treated with the catalytically inactive TaOGTK815M or with TaOGTWT in the absence of the donor substrate UDP-GlcNAc. hCK2α treated with hOGT(312–1031) was used as a positive control. WB, Western blot.

驱动由tubulin::GAL4. 儿龄F2后代被恢复从这一跨系确认 hOGT可以作为替补的母系要求的sxc在早期果蝇发育。活性TaoGT氨基酸，我们表达和纯化并从这一跨系确认 hOGT可以作为替补的母系要求的sxc在早期果蝇发育。活性TaoGT氨基酸，我们表达和纯化从这一跨系确认 hOGT可以作为替补的母系要求的sxc在早期果蝇发育。活性TaoGT氨基酸，我们表达和纯化
**FIGURE 3. TaOGT can rescue the lethality of Drosophila supersex combs (sxc) mutants.**

**a.** TaOGT WT restores O-GlcNAc levels in S2 cells lacking endogenous OGT. RNAi was used to knockdown endogenous OGT in S2 cells. GFP RNAi was used as a control. Cells were then transfected with plasmids carrying HA-tagged DmOGT, TaOGT WT, or catalytically inactive TaOGT K815M. Cells were lysed and total lysates were probed by Western blotting using the specified antibodies.

**b.** quantification of rescue to adulthood on driving DmOGT, TaOGT WT, or TaOGT K815M transgenes in sxc1/sxc6 mutants. The number of sxc transheterozygotes recovered on rescue with TaOGT WT is comparable with that of the DmOGT. The level of rescue with DmOGT is twice as that of TaOGT WT because the transgenic line used in the case of DmOGT was homozygous. O-GlcNAc levels in flies expressing TaOGT WT are comparable with those expressing DmOGT. Total lysates from w1118 (wt), rescued F1 sxc1/sxc6 transheterozygotes, or F2 sxc/sxc flies expressing HA-tagged UAS::DmOGT or UAS::TaOGT WT under the control of tubulin::GAL4 were probed by Western blotting using the specified antibodies.

**c.** O-GlcNAc in the Simplest Known Animal

**TABLE 1**

**Rescue of sxc lethality by TaOGT**

Crosses were set up with flies of the indicated genotypes and transferred into fresh vials every 3–4 days. Adults emerging from the crosses were scored for the presence of second and third chromosome balancers/marker, CyO and MKRS or TM6. Flies that did not possess any of the balancers/markers (±; ±) were the rescued sxc1/sxc6 transheterozygotes. Control crosses with flies lacking either the driver (tubulin::GAL4) or any of the OGT transgenes do not yield any non-CyO adults.

<table>
<thead>
<tr>
<th>Parental cross</th>
<th>Total adults</th>
<th>CyO; TM6</th>
<th>CyO; MKRS</th>
<th>CyO; MKRS/TM6</th>
<th>CyO; +</th>
<th>+; +</th>
</tr>
</thead>
<tbody>
<tr>
<td>sxc1/CyO; tub::GAL4/TM6 × sxc1/CyO;MKRS/TM6</td>
<td>119</td>
<td>26</td>
<td>54</td>
<td>39</td>
<td>NA*</td>
<td>0</td>
</tr>
<tr>
<td>sxc1/CyO;MKRS/TM6 × sxc1/CyO;UAS::TaOGT WT/TM6</td>
<td>195</td>
<td>54</td>
<td>97</td>
<td>44</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>sxc1/CyO;MKRS/TM6 × sxc1/CyO;UAS::TaOGT K815M/TM6</td>
<td>203</td>
<td>120</td>
<td>NA</td>
<td>NA</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>sxc1/CyO;MKRS/TM6 × sxc1/CyO;UAS::TaOGT WT/TM6</td>
<td>131</td>
<td>94</td>
<td>NA</td>
<td>NA</td>
<td>37</td>
<td>0</td>
</tr>
</tbody>
</table>

* NA, not applicable.
300-fold decrease in $k_{cat}$ of $TaOGA_{53}$ compared with $TaOGA_{54}$ is comparable with the reduced catalytic activity of the short versus full-length isoforms of hOGA reported previously (78, 79). The activities of both $TaOGA_{53}$ and $TaOGA_{54}$ are inhibited by the well characterized OGA inhibitors GlcNAcstatin C (80, 81) and Thiamet G (82) (Fig. 4, c and d). To further confirm that $TaOGA_{53}$ and $TaOGA_{54}$ are active, in vitro O-GlcNAcylated hCK2 and HEK293/Drosophila S2 cell lysates were treated with increasing amounts of the enzymes and probed for O-GlcNAc using the RL-2 antibody. Samples were also treated with the catalytically inactive $TaOGA_{53}^{D120A}$ and $TaOGA_{54}^{D121A}$. Treatment of samples with $TaOGA_{53}^{WT}$ did not lead to a noticeable decrease in O-GlcNAc signal (Fig. 5, a and c). Under the same experimental conditions, treatment with $TaOGA_{54}^{WT}$, but not its inactive counterpart $TaOGA_{54}^{D121A}$, resulted in a dose-dependent decrease in O-GlcNAc signal obtained in comparison to untreated controls (Fig. 5, b and d). To determine specificity of RL-2 to O-GlcNAc, lysates were also independently probed with RL-2 antibody preincubated with 0.5 M GlcNAc or secondary antibody alone. The difference in activity observed for $TaOGA_{53}$ and $TaOGA_{54}$ on lysates is not unexpected given the kinetic parameters of these enzymes. The ability of $TaOGA_{54}$ to de-O-GlcNAcylate HEK293 and S2 cell lysates demonstrates that Trichoplax possesses a functional orthologue of metazoan OGA.

Trichoplax Possesses O-GlcNAcylated Proteins—Having established that Trichoplax expresses functional orthologues of metazoan OGA.
of OGT and OGA, we investigated the presence of O-GlcNAcylated proteins in the organism by Western blotting using the anti-O-GlcNAc antibody CTD110.6. Trichoplax lysates were probed for O-GlcNAc alongside lysates of R. salina, the algal food source used to culture Trichoplax, a negative control. Trichoplax lysates showed reactivity toward the antibody, whereas Rhodomonas lysates did not, confirming that CTD110.6 reactive proteins were exclusively of Trichoplax origin (Fig. 6a).

Specificity of the signal toward O-GlcNAc was determined by preincubating CTD110.6 with 0.5M GlcNAc, which competed away CTD110.6 reactivity (Fig. 6b). The presence of O-GlcNAcylated proteins in Trichoplax was further confirmed using the alternative “Click-It” approach, whereby O-GlcNAc residues are labeled using a mutant galactosyltransferase (Gal-T1 Y289L) (83) with azido-modified galactose, which is then reacted with biotin-alkyne via copper-dependent cycloaddition and detected by Western blotting using peroxidase-conjugated streptavidin (84).
However, the Click-It method could potentially identify any glycosylated protein containing a terminal GlcNAc. *Trichoplax* lysates were therefore treated with PNGase F or *CpNagJ*, a bacterial OGA (51), to specifically remove N-linked glycans or O-GlcNAc, respectively, prior to performing the Click-It reactions, to ensure specific detection of O-GlcNAc. Although PNGase F treatment did not result in significant reduction in signal obtained with streptavidin-HRP, *CpNagJ* treatment led to a reduction in signal, establishing the specificity of the results obtained with the Click-It method (Fig. 6c). PNGase F-treated lysates were also probed with the lectin concanavalin A (ConA) to confirm activity of PNGase F on the N-glycans of *Trichoplax*. The specificity of ConA was assessed by competing it with 0.5 M α-methyl mannose pyranoside.

**DISCUSSION**

Our data show that the basal metazoan *Trichoplax* expresses functional OGT and OGA and also possesses O-GlcNAcyalted proteins. Our results suggest that OGT may have a ubiquitous role in *Trichoplax* because its transcripts do not exclusively localize at specific regions of the organism. It is remarkable, given the minimalist morphology of *Trichoplax*, that *TaOGT* is able to rescue the lethality of *Drosophila* OGT null mutants.
This suggests roles for OGT and protein O-GlcNAcylation in evolutionarily conserved processes in *Trichoplax*.

*Trichoplax* is unusual among metazoa in that it encodes two orthologues of OGA. It is possible given the biochemical properties of the shorter *Ta*OGA53 that this is an inactive enzyme with regulatory or scaffolding functions. Although the *K_m* for *Ta*OGA54 toward the pseudosubstrate 4MU-NAG is about 25-fold higher compared with hOGA (85), its activity on human cell lysates is comparable with that reported for hOGA previously (78), indicating functional conservation of OGA activity throughout metazoan evolution.

The phylogenetic position of *Trichoplax* at the base of metazoa has allowed it to be used in studies investigating the evolution of human cellular pathways and proteins (86, 87). A recent study used comparative genomics to identify metazoan-specific genes defined on the basis of being present in all metazoa including *Trichoplax*, but being absent in other eukaryotes (88). Its presence in *Trichoplax* but not in protists, adds *oga* to this repertoire of metazoan-specific genes. Although the presence of active OGT and O-GlcNAcylated proteins in plants and protists (55, 89) implies a eukaryote-specific role for intracellular O-GlcNAcylation, it is possible that the post-translational modification is dynamic and reversible only in metazoa. Alternatively, an as yet undiscovered enzyme with low sequence homology to OGA may be responsible for O-GlcNAc hydrolysis in protists and plants.

Signal transduction, in part through some types of post-translational modifications of proteins is thought to be one of the prerequisites for multicellularity in metazoa (88, 89). A recent study found higher levels of tyrosine phosphorylation in the proteome of *Trichoplax* than present in more basal or complex organisms, and ascribed it to the appearance of dedicated tyrosine kinases at the onset of metazoan multicellularity (67). Similarly, the acquisition of OGA by *Trichoplax* (and other metazoa) may have enabled the fine-tuning of signal transduction partly via facilitating interplay between O-GlcNAcylation and phosphorylation that is known to exist in other organisms (3–5).

We show that *T. adhaerens*, the simplest known animal, is a suitable reductionist model, as it is the most basal organism to possess the machinery required for reversible protein O-GlcNAcylation. Despite lacking the genetic tractability of other model systems, *Trichoplax* presents as a useful system to identify conserved O-GlcNAc proteins and help shape our understanding of the evolutionary roles for reversible O-GlcNAcylation.

Acknowledgments—We thank Leo Buss for providing *T. adhaerens* and *R. salina* and for advice on maintaining them in culture. We thank Bernd Schierwater for advice with performing in situ hybridization experiments.

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O-GlcNAc in the Simplest Known Animal

Glycobiology and Extracellular Matrices: The Early Metazoan Trichoplax adhaerens Possesses a Functional O-GlcNAc System

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