The *Drosophila* Neurally Altered Carbohydrate Mutant Has a Defective Golgi GDP-Fucose Transporter

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**Keywords:** Congenital disorders of glycosylation, *Drosophila*, Fucosylation, GDP-fucose transporter, Glycobiology, Glycoprotein biosynthesis, Mutant, N-glycosylation, HRP epitope, Sugar transport

**Background:** The defect underlying reduced HRP epitope expression in *Drosophila* nac mutants has not been identified.

**Results:** nac flies have a defective GDP-fucose transporter.

**Conclusion:** The defective nac transporter cannot support normal N-glycan core fucosylation, leading to reduced HRP epitope expression.

**Significance:** nac flies are a valid model for the human congenital disorder of glycosylation, CDG-IIc, which is caused by a similar molecular defect.

**SUMMARY**

Studying genetic disorders in model organisms can provide insights into heritable human diseases. The *Drosophila* neurally altered carbohydrate (nac) mutant is deficient for neural expression of the HRP epitope, which consists of N-glycans with core α1,3-linked fucose residues. Here, we show that a conserved serine residue in the Golgi GDP-fucose transporter (GFR) is substituted by leucine in nac flies, which abolishes GDP-fucose transport *in vivo* and *in vitro*. This loss of function is due to a biochemical defect, not to destabilization or mis-targeting of the mutant GFR protein. Mass spectrometry and HPLC analysis showed that nac mutants lack not only core α1,3-linked, but also core α1,6-linked fucose residues on their N-glycans. Thus, the nac Gfr mutation produces a previously unrecognized general defect in N-glycan core fucosylation. Transgenic expression of a wild-type Gfr gene restored the HRP epitope in neural tissues, directly demonstrating that the Gfr mutation is solely responsible for the neural HRP epitope deficiency in the nac mutant. These results validate the *Drosophila* nac mutant as a model for the human congenital disorder of glycosylation, CDG-IIc (also known as LAD-II), which is also the result of a GFR deficiency.

Congenital disorders of glycosylation (CDG’s) are a phenotypically diverse group of heritable diseases caused by mutations in genes functioning in glycosylation (recently reviewed by 1–3). The first congenital disorders that were recognized to have altered glycosylation patterns were identified in humans (4–7). These disorders were later found to involve deficiencies in N-glycan biosynthesis and processing (8–11) that were caused by mutations in genes encoding those functions (12–15).
The first CDG reported in a non-human organism was nac (neurally altered carbohydrate), which was identified in Drosophila. nac mutants have reduced levels of a neural carbohydrate epitope that is produced by α1,3-linkage of a fucose residue to the N-glycan core (16-18). Due to its identification as a dominant epitope in the plant glycoprotein horseradish peroxidase, this core α1,3-fucosylated N-glycan is also known as the horseradish peroxidase (HRP) epitope (19,20).

In Drosophila, the HRP epitope is expressed mainly in the central nervous system (CNS, 21-23), where it is produced by a fucosyltransferase designated FucTA (24-26). FucTA is a Golgi resident enzyme that transfers fucose from the donor substrate GDP-fucose to the proximal N-acetylgalactosamine residue of N-glycans (Fig. 1A). GDP-fucose is produced in the cytoplasm (27) and transported into the Golgi apparatus by GFR, a specific GDP-fucose transporter, in exchange for GMP (Fig. 1A, 28,29). The Drosophila Gfr gene is homologous to the human Gfr gene, which is defective in a congenital disorder of glycosylation known as CDG-IIc and also known as Type II leukocyte adhesion deficiency (LAD-II, 30,31) or SLC35C1-CDG, (32).

The original nac mutant, which was later re-designated nac1 to distinguish it from other alleles, has a temperature-insensitive loss of the neural HRP epitope associated with other cold-sensitive phenotypes expressed at 18°C, but not 25°C (33,34). Katz and coworkers cytogenetically mapped the nac1 mutation to the region between 84F4 and 84F11-12 (33), which includes about 32 genes. Subsequent work showed that the Gfr gene, which encodes a Golgi GDP-fucose transporter, is located in this region (28). This finding hinted that a Gfr mutation might be responsible for nac1 because a defect in the ability to transport GDP-fucose into the Golgi apparatus could account for the reduced neural HRP epitope in the nac1 fly. This speculation was strengthened by data showing that homozygous Gfr knockout flies have temperature-sensitive Notch-like wing phenotypes (35), which are similar to the temperature-sensitive scalloped wing phenotype observed in the nac1 mutant (34). However, neither the gene(s) mutated in nac1 flies nor the precise nature of the mutation have been elucidated. Thus, we examined the Gfr gene in the Drosophila nac1 mutant and found that nac1 flies have a mutant Gfr gene, which encodes a defective Golgi GDP-fucose transporter that is solely responsible for its neural HRP epitope deficiency.

**EXPERIMENTAL PROCEDURES**

*Genomic DNA Analysis—nac1* homozgyous flies were obtained from the Bloomington Drosophila Stock Center (Indiana University) and maintained at 28°C. Genomic DNA was extracted from a single adult fly, as previously described (36). Briefly, the fly was homogenized in a lysis buffer containing RNase A and the homogenate was incubated at 55°C for 1 h. The lysate was briefly centrifuged to remove debris and the DNA was precipitated. The DNA was dissolved in TE buffer, extracted once with phenol-chloroform, and 1 µl of the resulting DNA preparation was used as the template for a PCR with primers that flanked the Gfr gene transcript (AAGGGATGCGGCAAGAAGC and AATCCACCCCCGACTCAAC). All PCRs were performed using Phusion™ DNA polymerase (New England Biolabs). Agarose gel electrophoresis showed that the PCR yielded a single major amplification product of the expected size, which was gel-purified using the Qiaquick gel purification kit (Qiagen) and directly sequenced with the primers used for the PCR.

*Expression plasmids and baculoviruses—* All plasmid constructs derived directly from PCRs were sequence verified and amplimers for TOPO cloning were gel purified and then treated with Taq DNA polymerase (NEB). Total RNA was isolated from the Drosophila wt Canton-S strain using TRIzol® reagent (Invitrogen) and cDNA was synthesized using SuperScript® III RT (Invitrogen) and oligo-dT. The cDNA was used as the template to amplify the wt Gfr ORF (primers TCAGGCCCTCTGGAAGGCGGCTGCT and CACCATGACTAAACTGAGGGAGAC), which was cloned into pcDNA™3.1/V5-His-TOPO® (Invitrogen), and a sequence-verified clone was designated pcDNA-DmGFR-wt. This plasmid was used as the template for PCR mutagenesis with the additional primers GTGCACCTTGTATTGACGCTTACG and GTCAATATGAGTTGACCTAGAGAGC, which were designed to excise Gfr ORF by overlap PCR. The amplimer was cloned into pcDNA™3.1/V5-His-
TOPO®, and a clone encoding the nac
mutant Gfr was designated pcDNA-DmGFR-nac.
Baculovirus transfer plasmids were produced by cloning the BamHI-NotI fragment encoding the wt or nac
mutant Gfr gene from pcDNA-DmGFR-wt and pcDNA-DmGFR-nac-, respectively, into the BglII-NotI sites of pAcp(+)-IE1TV3 (37), resulting in production of the pAcp(+)-DmGFR-wt and pAcp(+)-DmGFR-nac transfer plasmids, respectively.

Plasmids encoding C-terminally eGFP-tagged GFR proteins were constructed by PCR overlap extension. The wt or nac
mutant Gfr ORFs minus their stop codons were PCR amplified using the respective pcDNA plasmids as the template, respectively, with the primers
CACCATGTACAAGAATCTGGAGGAGCAC
and CTACTTGTACAGCTCGTCCATGC. The eGFP ORF was PCR amplified using pcGFP-N1 (Clontech) as the template with the primers
CACCATGTACAAGAATCTGGAGGAGCAC
and GCTCACCATTGCTTCTGGGTGCGGT .
The reaction products were gel-purified and used as the template in PCR overlap extension reactions. The reaction products were cloned into pcDNA™3.1/V5-His-TOPO®, and clones encoding the C-terminally GFP-tagged wt and nac
mutant Gfr were designated pcDNA-DmGFR-wt-GFP and pcDNA-DmGFR-nac-GFP, respectively. The fused ORFs were excised from these plasmids using BamHI and NotI and cloned into the BglII-NotI sites of pAcp(+)-IE1TV3 (37), yielding pAcp(+)-IE1TV3-DmGFR-wt-GFP and pAcp(+)-IE1TV3-DmGFR-nac-GFP, respectively.

Transfer plasmids were used to produce recombinant baculoviruses by a standard allelic transplacement method (38,39) with BestBac viral expression plasmids encoding RFP-tagged S. frugiperda MGAT1 (44) and GFP-tagged wt or nac
mutant GFR proteins [pAcP(+)DmGFR-wt-GFP or pAcP(+)DmGFR-nac-GFP], plated on Concanavalin A coated dishes and photographed essentially as described before (43), except that biotin-conjugated AAL lectin (Vector labs) was used as the probe.

**Subcellular localization—Sf9 and Drosophila S2 cells** were transfected with expression plasmids encoding RFP-tagged S. frugiperda MGAT1 (44) and GFP-tagged wt or nac
mutant GFR genes using Lipofectamine™ 2000 (Invitrogen). Briefly, cells were transfected using 30 µg of DNA and 75 µl of transfection reagent for 3 h using serum-free αMEM according to the manufacturer’s protocol. Cells were subsequently incubated for 24 h in αMEM with 15% FBS, after which cells were collected by trypsinization, washed twice in PBS and lysed in 500 µl lysis buffer (20 mM Tris-HCl, pH 7.4, 1.0% NP-40, 150 mM NaCl, 0.5 mM PMSF, 1 mM EDTA, one Complete Mini™ protease inhibitor cocktail tablet (Roche) / 10 ml buffer). After vigorous vortexing, the lysate was centrifuged for 10 minutes at 13,000 g, after which the supernatant was collected and assayed for soluble protein concentration using a commercial BCA assay (Pierce).

**CHO cell lysates** were prepared as described above from CHO cells cultured as described before (41). Aliquots containing 50 µg of total protein were separated by SDS-PAGE (42) and transferred to an Immobilon-P PVDF membrane (Millipore). The membrane was blocked, probed and developed essentially as described before (43), except that biotin-conjugated AAL lectin (Vector labs) was used as the probe.

**GDP-fucose transport assays**—Fifty ml of Sf9 cells were seeded at a density of 5 x 10³ cells/ml in complete TNM-FH medium, grown overnight to 1 x 10⁶ cells/ml and infected with the appropriate viral stock at a multiplicity of infection of about 2 plaque-forming units/cell. After 22 h, infected cells were pelleted at 500 g for...
then adding 10 µl STM buffer containing 30 nCi $^{3}H$-GDP-fucose, (Fucose-2-$^{3}H$N), Perkin-Elmer Life Sciences; 15-35Ci/mmol). The mixture was then centrifuged at 13,000 g for 10 minutes and the concentration of solubilized protein in the supernatants were determined using a commercial BCA assay (Pierce).

For transport assays, aliquots of the microsomal preparations were thawed on ice, thoroughly vortexed, and transport assay mixtures were prepared by adding 10 µl of the microsomal preparation to 80 µl STM buffer, cooling the mixtures to a water bath at 18°, 25° or 32°C for precisely 1 min, returned to the ethanol-ice bath and quenched by the addition of 900 µl ice-cold STM buffer. The mixtures were then filtered through water-wetted 0.45 µm mixed cellulose esters filters (Type HA; Millipore) using a 1225 Sampling Manifold (Millipore). The disks were washed three times with 5 ml of ice-cold STM buffer, air-dried, placed in 7 ml of Ultima Gold F scintillation cocktail (Packard Instrument Company), and counted for 10 min in a Model LS-6500 liquid scintillation spectrometer (Beckman Coulter).

Background counts were determined by counting an unused filter as described above. All samples were assayed at least three times in triplicate (n=9). Raw counts were corrected for background and normalized to 30 µg of soluble protein content. Significant differences were determined by one-way ANOVA using Microsoft® Excel.

**Mass-spectrometry and HPLC**—For nac$^{1}$ and wt Canton S flies, pepsin glycopeptides were enriched and N-glycans were released with PNGase A prior to pyridylamination and RP-HPLC, MALDI-TOF MS or ESI-MS analysis (45). As a first step, linear MALDI-TOF mass spectra of unlabeled N-glycans were obtained prior to pyridylamination using a Thermo Bioanalysis Dynamo mass spectrometer in linear mode with 2,5-dihydroxybenzoic acid as matrix. For ESI-MS of the pyridylaminated glycans with a Micromass Q-TOF Ultima Global mass spectrometer, the [M+H]$^{+}$ ions were calculated by applying the MassLynx MaxEnt3 software to the raw multiply-charged ion data. For reversed-phase HPLC analysis of pyridylaminated N-glycans, an ODS Hypersil column (250 x 4 mm) with a gradient of 0.3% methanol per minute was used with an oligohexose series (3-11 glucose units) as a calibration standard; elution times in terms of glucose units can be compared to previous data on wt fly N-glycans (24). Individual RP-HPLC fractions were also analyzed by MALDI-TOF MS and MS/MS using a Bruker autoflex™ speed instrument in reflectron mode and 6-aza-2-thiothymine as matrix.

**Drosophila transgenesis and rescue**—All Drosophila strains (OreR, w$^{1118}$, elav-GAL4 inserted on the X chromosome, nac$^{1}$, and balancer stocks) were obtained from the Bloomington Drosophila Stock Center. The full wt Canton S Gfr ORF was isolated by PCR using the primers GGAATTCCGAAATGTACAAGAATCTG and GGGGACCCTCAGGCCCTTCTGGTG. The amplicon was purified and cut with EcoRI and KpnI and ligated into the same sites of the pUAST transgenesis plasmid (46). Transgenic stocks carrying UAS-Gfr elements on all three chromosomes were generated by injection of...
pUAST-Gfr into pre-cellularized embryos using standard methods (46).

Drosophila embryo anti-HRP staining—Embryos were dechorionated, fixed, devitellinized, stained with antibodies and staged according to standard methods (47,48). Antibody dilutions were 1:5000 for rabbit anti-HRP (Jackson Immunoresearch) and 1:2000 for peroxidase-conjugated secondary antibodies (Jackson Immunoresearch). All embryos were processed identically and in parallel (same antibody dilutions, same development time, same day) to facilitate objective comparison of HRP epitope levels in all genotypes.

RESULTS

Gfr Gene Sequence in the nac1 Mutant—The sequence of a PCR amplimer from a genomic region that includes the Gfr transcript revealed that the Gfr gene in the nac1 mutant has a single mutation consisting of a cytosine to thymidine transition at position 86 (C86T) of the ORF. This mutation was independently identified in the Jarvis and Wilson labs in nac1 stocks obtained at different times and from different sources. The nac1 C86T transition results in the substitution of a leucine for a serine residue at position 29 (S29L) in the predicted GFR amino acid sequence. The wild type (wt) serine residue is fully conserved among all known and putative GDP-fucose transporters throughout the animal kingdom (Fig. 1B) and is located in the first predicted transmembrane region (Fig. 1C). As production of the HRP epitope requires GDP-fucose in the Golgi apparatus, where it serves as the donor substrate, these observations were consistent with the idea that nac1 flies might have a defective Golgi GDP-fucose transporter. Thus, we examined the impact of the nac1 S29L mutation on the GDP-fucose transport function of the mutant Gfr gene product.

GDP-fucose Transport by the nac1 mutant GFR product—Our transport assays measured the amount of GDP-fucose transported into microsomes, analogous to previously described nucleotide-sugar transport assays employing Golgi-enriched microsomes from cells expressing heterologous NSTs (35,49). Baculovirus expression vectors were used to express the wt and nac1 mutant GFRs in S9 insect cells and then Golgi-enriched microsomes were isolated from those cells and used for GDP-fucose transport assays. Microsomes from cells infected with the empty baculovirus vector were used as background controls and each assay was performed at different temperatures to determine if there were any temperature-dependent differences that could explain nac1 cold sensitive phenotypes (34). As compared to the background controls, microsomes containing wt GFR imported more and those containing the nac1 mutant GFR imported less GDP-fucose at all temperatures examined (Fig. 2A). Increasing the assay temperature from 18°C to 25°C did not produce a statistically significant increase in GDP-fucose import in either the background controls or wt GFR samples. However, this temperature shift significantly increased GDP-fucose import in the nac1 GFR samples. These experiments were extended by performing additional assays at 32°C to determine if the nac1 mutant transporter gained even more function at this higher temperature. Indeed, GDP-fucose import with background control, wt GFR, and nac1 GFR microsomes was increased further at 32°C (Fig. 2A). As with the previous temperature shift, the increase in GDP-fucose import activity was highest in the nac1 GFR samples, confirming that the nac1 mutant GFR is more cold sensitive than wt GFR, which potentially contributes to the cold sensitive nac1 phenotypes (34).

We also assessed the function of nac1 GFR in vivo by using cells from a CDG-IIc (LAD-II) patient (40). These cells cannot produce fucosylated N-glycans because they have a defective GFR, but their fucosylation-negative phenotype can be rescued by transfection with a wt human Gfr gene (28,30,31). Thus, we transfected CDG-IIc (LAD-II) cells with plasmids encoding the wt or nac1 Drosophila Gfr genes, prepared total cell lysates and CHO cell lysates as a positive control, and probed them with the fucose-specific lectin AAL (50). AAL bound strongly to multiple proteins in the CHO cell lysate, but not to any proteins in the empty vector-transfected CDG-IIc (LAD-II) cell lysate, as expected (Fig. 2B). AAL also bound to multiple proteins in the wt Gfr-transfected CDG-IIc (LAD-II) cell lysate, but not to any proteins in the nac1 Gfr-transfected CDG-IIc (LAD-II) cell lysate (Fig. 2B). The higher level of AAL binding observed with the CHO cell lysate as compared to the wt Gfr-transfected CDG-IIc (LAD-II) cell lysate...
probably reflects cell toxicity associated with the transfection, as we observed significant cell death at later time points. Alternatively, it might reflect the inefficiencies inherent in the transfection process, or the differences between the CHO and LAD-II cell types. Regardless, these results showed that the nac1 Gfr gene failed to rescue the fucosylation-negative phenotype in CDG-IIc (LAD-II) cells, indicating that the nac1 mutant gene product is defective in vivo.

The nac1 Mutant GFR Localizes to the Golgi Apparatus—GDP-fucose transporters typically localize to the Golgi apparatus (28,40). Hence, the transport defect observed with the nac1 mutant gene product could have resulted from a direct impact of the mutation on its biochemical function or an indirect impact on its intracellular trafficking. To distinguish between these possibilities, we expressed GFP-tagged forms of GFR and MGAT1 as a Golgi marker, as this enzyme acts immediately downstream of the GDP-fucose transporters (44). Hence, the transport defect observed with the nac1 mutant gene product is defective in vivo.

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were crossed to generate embryo collections, which were then stained with the anti-HRP antibody to assess whether transgenic Gfr expression could rescue the nac\(^1\) core α1,3-fucosylation defect. As expected for the neural specificity of the elav-GAL4 element (56), expression of the HRP-epitope was rescued in differentiating neurons of elav-GAL4; UAS-Gfr; nac\(^1\)/nac\(^1\) progeny (Fig. 4C and D). Thus, reduced HRP epitope expression in nac\(^1\) flies is due solely to a defect in their Gfr gene.

Staining with anti-HRP antibody could be detected in late stage 10 rescued embryos, which is substantially earlier than in wild-type embryos, where staining first appeared in early stage 12. This is consistent with the time course of elav expression (56), suggesting that Gfr expression at least partially limits core α1,3-fucosylation in Drosophila. Surprisingly, elav-driven expression of Gfr resulted in embryonic lethality at mid-embryogenesis. This is likely a result of our use of the very strong elav-GAL4 driver, which typically provides highly efficient expression of UAS-transgenes in the embryonic nervs system.

In the course of generating UAS-Gfr transgenic stocks, we identified a line that exhibited partially rescued HRP-epitope expression in the ventral nerve cord and peripheral nervous system of nac\(^1\) mutant embryos without crossing to a GAL4 driver line (data not shown). This leaky expression line (UAS-Gfr\(^{vk2}\)) was homozygous viable and fertile in both nac\(^1\) and wt backgrounds, suggesting a significantly lower Gfr expression level than was obtained by crossing UAS-Gfr lines to the elav-GAL4 driver line. Interestingly, the UAS-Gfr\(^{vk2}\) leaky expression line rescued temperature-sensitive lethality associated with the nac\(^1\) mutant; only 6% of nac\(^1\)/nac\(^1\) adults survived after shift to 18°C, while 82% of UAS-Gfr\(^{vk2}\)/UAS-Gfr\(^{vk2}\); nac\(^1\)/nac\(^1\) adults survived and reproduced at 18°C. Thus, while overexpression of Gfr proved to be embryonic lethal, moderate expression was well tolerated and rescued both HRP-epitope expression and developmental arrest defects associated with the nac\(^1\) mutation.

**S29 is Conserved in Other GDP-sugar Transporters**—Finally, we compared the amino acid sequences of the Drosophila GFR and other known Golgi nucleotide sugar transporters (NSTs) to more generally assess the potential functional relevance of S29 (Fig. 5). We found that plant, fungal, protozoan, and animal GDP-sugar transporters have clear homology to an amino acid sequence in the N-terminal region of the Drosophila GFR. Several fungal Golgi GDP-mannose transporters (57-62), a Leishmania Golgi GDP-mannose, -fucose, and -arabinose transporter (63,64), and the Arabidopsis and Volvox Golgi GDP-mannose transporters (65-67) are clearly similar to Drosophila GFR in this region and each has a serine residue in positions corresponding to Drosophila GFR S29. Other multi-substrate transporters that also could transport a GDP-sugar, including human HFRC1 (68), Drosophila FRC (69), human UGTrel7 (70), and nematode SQV-7 (71) are similar to Drosophila GFR, as well, and each has a serine residue at a position corresponding to Drosophila GFR S29. In contrast to these GDP-sugar transporters, other Golgi NSTs including the human UDP-galactose (72), CMP-sialic acid (73), UDP-N-acetylglucosamine (74), and UDP-xylene transporters (75) lack significant homology to GFR. Of these, the human UDP-galactose and UDP-N-acetylglucosamine transporters have a serine residue at a position corresponding to Drosophila GFR S29. However, these serine residues are not conserved in the homologous transporters from most other species, indicating that, unlike Drosophila GFR S29, they are probably not essential for functionality. Based on these data, we suggest that a serine residue at a position corresponding to Drosophila GFR S29 in Golgi NSTs that also have similarity to an amino acid sequence in the first transmembrane domain of Drosophila GFR might predict GDP-sugar transport capacity.

**DISCUSSION**

Congenital disorders of glycosylation (CDG’s) are a diverse group of heritable diseases caused by mutations in genes involved in glycosylation. The study of CDG’s has been facilitated by the availability of animal models, as much of the glycosylation machinery is evolutionarily conserved (76,77). Since the description of the nac\(^1\) mutant in 1988 by Katz and coworkers (33), Drosophila has become established as a model organism for the study of human genetic disorders, including CDG’s (78,79). However, the genetic defect underlying the nac\(^1\) mutation had not yet been elucidated.
In this study, we identified a single nucleotide transition (C86T) that produces a leucine for serine substitution at position 29 of the Golgi GDP-fucose transporter encoded by the nac\(^1\) Gfr gene. The mutagen originally used to isolate the nac\(^1\) mutant was ethyl methane sulfonate (EMS, 33). Mechanistically, EMS is expected to produce G/C to A/T transitions (80,81), and this expectation has been confirmed in mutagenesis studies (82-84). Thus, the C86T transition in the nac\(^1\) Gfr gene is fully consistent with the use of EMS mutagenesis in producing the nac\(^1\) strain.

There are three currently known missense mutations in the human GDP-fucose transporter that cause CDG-IIc (LAD-II; T308R, R147C, Y337C, 30,85). In each case, these mutations alter a residue analogous to Drosophila GFR S29, which is fully conserved among animal GDP-fucose transporters and is located in a predicted transmembrane helix. Like these human GFR missense mutations, the nac\(^1\) S29L mutation also abolishes GDP-fucose transport function in vitro and in vivo. Serine residues corresponding to Drosophila GFR S29 are conserved in GDP-sugar transporters from a wide variety of species, but not in other types of NSTs. Thus, we speculate that a serine residue at a position corresponding to Drosophila GFR S29 might predict GDP-sugar transporting capacity in Golgi NSTs that are also otherwise similar to Drosophila GFR. Interestingly, the first TM domains of human HFRC1 and UGTrel7, like that of Drosophila FRC, are similar to GFR and each has a conserved serine corresponding to GFR S29, unlike any other human or Drosophila Golgi NSTs. Thus, it is possible that the low level of fucosylation in nac\(^1\) flies is due to GDP-fucose transport by the Drosophila FRC gene product. Similarly, it is possible that the alternative GDP-fucose transport activity observed in CDG-IIc (LAD-II) cells supplemented with fucose is due to this same function of the human HFRC1 or UGTrel7 gene products.

For our in vitro GDP-transport assays, we used microsomes from Sf9 cells (86), which have endogenous Golgi GDP-fucose transport activity, as these cells typically produce α1,6 core fucosylated N-glycans (87). Despite the presence of this endogenous activity, we were able to demonstrate a >6-fold increase in transport activity in microsomes from cells infected with a baculovirus encoding wt GFR, as compared to background controls. Surprisingly, microsomes from cells infected with a baculovirus encoding the nac\(^1\) GFR samples had reduced GDP-fucose import activity compared to the controls, indicating a possible dominant negative effect. Considering that GFR dimerization might be necessary to produce a functional transporter (29), co-expression of the nac\(^1\) GFR could have produced a subpopulation of heterodimers consisting of endogenous transporter molecules and recombinant nac\(^1\) GFR molecules, which were less functional than the endogenous transporter homodimers. A similar dominant negative phenotype in which co-expression of a mutant transporter negatively affects transport has been observed with the yeast Golgi GDP-mannose transporter (88), which also functions as a homodimer. Alternatively, it is possible that overexpression of the nac\(^1\) GFR altered the subcellular distribution of the endogenous transporter, thereby reducing the number of transporter molecules in those microsomal preparations.

Non-functional, mutant NSTs that fail to exit the ER typically have frameshift mutations that eliminate one or more transmembrane domains and the C-terminal domain. Two such mutations have been identified in the human GDP-fucose transporter (40,89). On the other hand, point mutations that change single amino acids typically do not alter the Golgi localization of NSTs, including the GDP-fucose transporter (31,40,90). Like the inactivating missense mutations in human GFR, the nac\(^1\) S29L mutation did not affect subcellular distribution, as both wt and nac\(^1\) GFR were Golgi localized.

ESI-MS, RP-HPLC and MALDI-TOF analyses demonstrated that nac\(^1\) flies have reduced levels of core α1,3-fucosylated and only trace levels of core α1,6-/α1,3-difucosylated N-glycans, which is consistent with the original observation that nac\(^1\) flies have significantly reduced levels of the HRP epitope. We also discovered that these flies had reduced levels of core α1,6-fucosylated N-glycans, which is consistent with the requirement of a Golgi GDP-fucose transporter for both core α1,6- and α1,3-fucosylation. The residual levels of monofucosylated N-glycans indicate that nac\(^1\) flies are still able to transport some GDP-fucose into the Golgi. This suggests
the presence of an alternative, functionally redundant GDP-fucose transport mechanism, a notion that is supported by the results of another study, in which faint anti-HRP and AAL staining could still be detected in flies with a large deletion in the Gfr gene (91). This redundant transport mechanism is not provided by the ER-localized GDP-fucose transporter encoded by the Efr gene, as flies lacking Gfr alone or both Gfr and Efr have comparable amounts of residual core fucosylated N-glycans (91). Similarly, humans also have an alternative, but less efficient Golgi GDP-fucose import mechanism, as dietary fucose supplementation can restore N-glycan core fucosylation in CDG-IIc (LAD-II) patients that are homozygous for a completely non-functional Golgi GDP-fucose transporter (40,89). The precise nature of the redundant GDP-fucose transport mechanism remains to be determined in both humans and flies, however, its low-affinity and non-saturable character suggests it is not provided by another specific GDP-fucose transporter (92,93).

Interestingly, nac<sup>1</sup> embryos rescued with elav-driven wt Gfr expressed the HRP epitope at earlier developmental stages than wt embryos, suggesting that N-glycan fucosylation is at least partially limited by transport of GDP-fucose into the Golgi apparatus. The notion that Gfr expression limits fucosylation in vivo is corroborated by the demonstration that increased N-glycan fucosylation in cancer cells correlates with increased Gfr expression, and that fucosylation can be increased directly by overexpressing Gfr (94). A surprising observation was that elav-driven wt Gfr overexpression triggered embryonic lethality. This is likely a pleiotropic phenotype arising from the increased availability of GDP-fucose in the Golgi apparatus for a variety of N-linked and O-linked fucosylation reactions. Intriguingly, the nac<sup>1</sup> phenotypes and the embryonic lethality observed in elav-GAL4; UAS-Gfr rescued nac<sup>1</sup> flies suggest that it is biologically necessary to maintain protein fucosylation within a certain range. Furthermore, the relation between Gfr expression levels, embryonic lethality, and HRP-epitope production suggests that Gfr is part of the regulatory system that maintains Golgi GDP-fucose levels within a physiologically acceptable range. Finally, the observation that transgenic wt Gfr expression can restore HRP epitope production in nac<sup>1</sup> flies indicates that the Gfr C86T transition is the only genetic defect responsible for the neurally altered carbohydrate phenotype. Hence, the defective Golgi GDP-fucose transporter and the resulting fucosylation deficit in nac<sup>1</sup> flies is analogous to human CDG-IIc (LAD-II). Coupled with our observation that the nac<sup>1</sup> mutant GFR is more cold-sensitive than its wt counterpart, we suggest that the nac<sup>1</sup> fly is a useful model of human CDG-IIc (LAD-II) that could be effectively exploited in a variety of creative ways, such as using its cold-sensitive phenotypes to titrate N-glycoprotein core fucosylation.

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FOOTNOTES

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2The abbreviations used are: AAL, Aleuria aurantia lectin; CNS, Central nervous system; ER, Endoplasmic reticulum; ESI-MS, Electrospray ionization mass spectrometry; NST, Nucleotide sugar transporter; RFP, red fluorescent protein; RP-HPLC, reverse phase HPLC; TM, Transmembrane.
**FIGURE LEGENDS**

**FIGURE 1. Production of the HRP epitope and the Drosophila nac\textsuperscript{1} GFR mutation.** A, GDP-fucose is produced in the cytoplasm and transported into the Golgi lumen by the GFR transporter in exchange for GMP. FucTA uses GDP-fucose in the Golgi lumen to produce the HRP epitope consisting of core α1,3-fucosylated N-glycans. Squares: N-acetylglucosamine; circles: mannose; triangles: fucose. B, Amino acid sequence comparison of known and predicted GDP-fucose transporters. The arrow indicates the conserved serine residue that is mutated to a leucine in Drosophila nac\textsuperscript{1} GFR. Amino acid residue numbering is according to the Drosophila gene product. Genbank accession numbers are: Drosophila: NP_649782.1; Mosquito: XP_312562.2; Honeybee: XP_623632.1; Flour beetle : XP_967192.1; Human: NP_001138737.1; Chinese hamster : BAE16173.1; Zebrafish: NP_001008590.1; Sea urchin: XP_798515.1; Nematode: XP_002637574.1. C, S29 is located in the middle of the first predicted transmembrane domain of Drosophila GFR.

**FIGURE 2. Wt, but not nac\textsuperscript{1} GFR can transport GDP-fucose in vitro and in vivo, and both are Golgi localized.** A, \(^3\)H-GDP-fucose import activity of Golgi-enriched microsomes from Sf9 cells infected with baculovirus vectors encoding wt GFR, nac\textsuperscript{1} GFR, or no exogenous transporter (−) at 18°, 25° or 32°C. Background import at 18°C (1.5 fmol GDP-fucose µg total protein\(^{-1}\) minute\(^{-1}\)) is set at 100% (−), error bars: 95% CI. p-values for different samples at the same temperatures were all <0.01. *: p<0.05; **: p<0.01. B, AAL lectin blot of CHO cells or CDG-IIc (LAD-II) cells transfected with expression plasmids encoding wt GFR, nac\textsuperscript{1} GFR, or nothing (−). C, Subcellular distribution of wt and nac\textsuperscript{1} GFR in Sf9 and Drosophila S2 cells. Columns: phase contrast, GFP-tagged GFR, RFP-tagged MGAT1 (insect Golgi marker), GFP and RFP merge, overlay. Scale bar, 10 µm.

**FIGURE 3. Core di-, α1,3- and α1,6-fucosylated N-glycan levels are strongly reduced in nac\textsuperscript{1} flies.** PNGase A-released N-glycans from wt Canton S (A, C, E, F) and nac\textsuperscript{1} adults (B, D, E, G) were subjected to analysis by ESI-MS (A-D), RP-HPLC (E) and MALDI-TOF MS (F,G). Abbreviations: F, fucose; G, glucose; M, mannose; N, N-acetylhexosamine. Symbols: red triangles, fucose; green circles, mannose; blue squares, N-acetylglucosamine. The late elution time of M3N2F (E) indicates it is a core α1,6-fucosylated glycan and its reduced relative intensity in nac\textsuperscript{1} flies is shown by all three methods, MALDI-TOF MS analysis (not shown) of individual RP-HPLC fractions indicated trace levels of difucosylated glycans co-eluting with M3N2 in nac\textsuperscript{1} flies. The exploded views of the ESI-MS spectra (C, D; m/z 1265-1335) are set to the same ion count (y-axis; 1.5 x 10\(^5\)) and show the almost complete absence in nac\textsuperscript{1} flies of the difucosylated HRP epitope MMF\(^3\)F\(^6\) glycan in its [M+H]\(^+\) and [M+Na]\(^+\) forms.

**FIGURE 4. Reduced HRP epitope expression in nac\textsuperscript{1} homozygous embryos is rescued by transgenic expression of wt Gfr.** A,C,E, lateral view. B,D,F, ventral view. All embryos are late stage 12 to early stage 13. In nac\textsuperscript{1}/nac\textsuperscript{1} embryos (A,B), HRP epitope expression is reduced in comparison to wt embryos (E,F). A wt Gfr transgene driven by the neural-specific elav promoter rescues neural HRP epitope expression (C,D). Scale bar, 70 µm.

**FIGURE 5. Drosophila GFR S29 is conserved in other GDP-sugar transporters.** Alignment of Drosophila GFR with other characterized GDP-sugar transporters from L. donovani (GDP-SugarT; 64), S. cerevisiae (Yeast GDP-ManT; 59), P. pastoris (Pichia GDP-ManT; 57), C. albicans (C. albicans GDP-ManT; 62), C. glabrata (C. glabrata GDP-ManT; 61), C. neoformans (Cryptococcus GDP-ManT-1 and -2; 58), A. nidulans (Aspergillus GMT-1 and -2; 60), V. carteri (Volvox GDP-ManT; 67), A. thaliana (Arabidopsis GONST-1 and -2; 65,66), and similar NSTs of C. elegans (Nematode SQV-7; 71), D. melanogaster (Drosophila FRC; 69), and humans (Human FRC1 and hUGTrel7; 68,70).
Figure 1

**A**

Cytoplasm

GDP-fucose

GFR

GMP

Golgi lumen

HRP epitope N-glycan

**B**

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**C**

Golgi lumen

13 45 117 121 163 173 227 242 289 298

N cytoplasm C
Figure 2

(A) Graph showing % of $^3$H DPM with bars for different temperatures and conditions.

(B) Image of protein gel with lanes for CHO, wt Gfr, LAD-II, and nac$^1$ Gfr.

(C) Stacked images showing phase, wt GFR, MGAT1, merge, and overlay for different stages (S6, S9, S2).
Figure 3

**A (wt)**
- 6.04 x 10^6
- 973.46
- 1135.51
- M2N2F
- M3N2F
- M3N2
- M5N2
- M9N2
- M9N2

**B (nac')**
- 15.0 x 10^6
- 973.46
- 1135.50
- M2N2F
- M3N2F
- M3N2
- M5N2
- M9N2
- M9N2

**C (wt)**
- 1.50 x 10^5
- 1281.60
- 1303.58
- M3N2
- M3N2F
- MMFF8

**D (nac')**
- 1.50 x 10^5
- 1281.59
- 1303.56
- M3N2
- M3N2F

**E**
- Fluorescence intensity vs. glucose units
- 3 4 5 6 7 8 9 10 11
- M9N2
- M5N2
- M3N2
- M3N2F

**F (wt)**
- M3N2
- M3N2F
- M3N2F
- M5N2
- M9N2
- M9N2

**G (nac')**
- M3N2
- M3N2F
- M3N2F
- M5N2
- M9N2

**M3N2F**
- 1080
- 1258
- 1420
- 1582
- 1744

**M3N2**
- 934
Figure 4

Drosophila neurally altered carbohydrate mutant

Lateral view

A

B

C

D

E

F

Ventral view

nac'/nac^1
elav-GAL4; UAS-Gfr;
nac'/nac^1

OreR
Figure 5

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The Drosophila neurally altered carbohydrate mutant has a defective Golgi GDP-fucose transporter
Christoph Geisler, Varshika Kotu, Mary Sharrow, Dubravko Rendic, Gerald Pöltl, Michael Tiemeyer, Iain B. H. Wilson and Donald L. Jarvis

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