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Drosophila UDP-glucose:glycoprotein glucosyltransferase: sequence and characterization of an enzyme that distinguishes between denatured and native proteins

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A *Drosophila* UDP-glucose:glycoprotein glucosyltransferase was isolated, cloned and characterized. Its 1548 amino acid sequence begins with a signal peptide, lacks any putative transmembrane domains and terminates in a potential endoplasmic reticulum retrieval signal, HGEL. The soluble, 170 kDa glycoprotein occurs throughout *Drosophila* embryos, in microsomes of highly secretory *Drosophila* Kc cells and in small amounts in cell culture media. The isolated enzyme transfers [^{14}C]glucose from UDP-[^{14}C]Glc to several purified extracellular matrix glycoproteins (laminin, peroxidasin and glutactin) made by these cells, and to bovine thyroglobulin. These proteins must be denatured to accept glucose, which is bound at endoglycosidase H-sensitive sites. The unusual ability to discriminate between malfolded and native glycoproteins is shared by the rat liver homologue, previously described by A.J.Parodi and coworkers. The amino acid sequence presented differs from most glycosyltransferases. There is weak, though significant, similarity with a few bacterial lipopolysaccharide glycotransferases and a yeast protein Kre5p. In contrast, the 56–68% amino acid identities with partial sequences from genome projects of *Caenorhabditis elegans*, rice and *Arabidopsis* suggest widespread homologues of the enzyme. This glucosyltransferase fits previously proposed hypotheses for an endoplasmic reticular sensor of the state of folding of newly made glycoproteins.

Key words: denaturation/glucosyltransferase/glycoprotein processing/protein folding/transient glucosylation

Introduction

Peptide synthesis of secreted glycoproteins is followed by two concurrent modification processes within the endoplasmic reticulum (ER). One is polypeptide folding and assembly of multichain proteins. The other is the construction and modification of N-linked oligosaccharides. Although overall interrelationships exist between progression along the folding and glycosylation paths, there is little understanding of the underlying molecular mechanisms that might couple the two processes. Here we report on a potential component of such a connection: an ER glucosyltransferase that attaches glucose to malfolded, but not to native glycoproteins. This may provide a recognition tag for chaperone systems that assist newly synthesized proteins in acquiring their final, native conformation (Sousa *et al.*, 1992; Hammond and Helenius, 1993; Hammond *et al.*, 1994).

The presence of a single terminal glucose residue on N-linked oligosaccharides of newly synthesized polypeptides has been correlated with the need for assistance in protein folding. In particular, several viral mutant proteins that do not exit the ER because of malfolding or improper assembly have been demonstrated to be monoglucosylated (Rizzolo and Kornfeld, 1988; Suh *et al.*, 1989). N-linked oligosaccharides are synthesized in most eukaryotes from a high mannose dolichol-linked precursor that contains three glucose residues. The transfer of this precursor to Asn in the nascent polypeptides and trimming of the glucose moieties by ER enzymes glucosidase I (which removes the terminal α1,2 linked glucose) and glucosidase II (which removes both of the inner α1,3 linked glucose residues) are established (reviewed by Kornfeld and Kornfeld, 1985). Although the single glucose residue of $Glc_1Man_9GlcNAc_2$ is an intermediate of this trimming process, it is also formed by re-glucosylation of $Man_9GlcNAc_2$ by an ER glucosyltransferase (Parodi *et al.*, 1983). Parodi and co-workers characterized this post-translational glucosylation reaction and called it transient glucosylation, because the added glucose residues are rapidly trimmed, probably by glucosidase II. Pulse–chase experiments with labelled UDP-glucose demonstrated that transient glucosylation occurs in calf thyroid, rat liver, plants, trypanosomatids (Parodi *et al.*, 1983, 1984a,b), fungi (Lederkremer and Parodi, 1984) and COS cells (Suh *et al.*, 1989). An *in vitro* assay was developed to detect glucosylation of high mannose glycoproteins using bovine thyroglobulin as a test substrate (Trombetta *et al.*, 1989) and the activity was found in microsomal fractions from several organisms.

The enzyme responsible for the re-glucosylation activity, UDP-Glc:glycoprotein glucosyltransferase, was purified to homogeneity and characterized from rat liver microsomes (Trombetta and Parodi, 1992). This protein is itself a glycoprotein, with an apparent monomeric M_r of 150 kDa. It is the only glycosyltransferase within the ER which is not membrane bound (Trombetta *et al.*, 1989, 1991). UDP-Glc:glycoprotein glucosyltransferase adds glucose from UDP-glucose to high mannose glycoproteins in the presence of Ca^{2+} ions and the resulting glucosylated oligosaccharide has the same structure as the processed intermediate, $Glc_1Man_9GlcNAc_2$ (Trombetta *et al.*, 1989). An interesting characteristic of the enzyme is that unfolded, denatured glycoproteins are substantially better substrates for glucosylation than are the corresponding native proteins, even though the terminal mannose residues to which glucose becomes linked are equally accessible in the

native and denatured forms. Furthermore, glucosylation of denatured glycoproteins is competitively inhibited by addition of de-glucosylated, denatured proteins. Somehow this enzyme has an ability to discriminate the state of folding of a glycoprotein, effectively transferring glucose only to imperfectly folded proteins. This led to the suggestion that both the enzyme and transient glucosylation are involved in key processes of monitoring and/or assisting the folding and assembly of newly made glycoproteins, possibly in association with the membrane-bound chaperone calnexin (Sousa *et al.*, 1992; Hammond *et al.*, 1994). A recent report suggests that calnexin binds to monoglucosylated glycoproteins, which may be the product of transient glucosylation (Hammond *et al.*, 1994).

Drosophila extracellular matrix contains several large glycoproteins, such as laminin, that share glycosylation, folding and assembly problems with their vertebrate counterparts. The *Drosophila* cell line Kc 7E10 secretes substantial amounts of highly glycosylated extracellular matrix proteins as well as other large glycoproteins into the culture media (Fessler *et al.*, 1994). Our studies of these cells led to the isolation and characterization of the protein that we describe here: *Drosophila* UDP-Glc:glycoprotein glucosyltransferase (DUGT). The cDNA encoding this protein was found to have small, but significant sequence homology to a family of bacterial lipopolysaccharide glycosyltransferases. The isolated protein was demonstrated to transfer glucose to several *Drosophila* extracellular matrix proteins, also synthesized in Kc 7E10 cells, and to bovine thyroglobulin in the same manner as the UDP-Glc:glycoprotein glucosyltransferase isolated from rat liver. The deduced amino acid sequence suggests that this is a novel glucosyltransferase.

Results

Isolation and characterization of DUGT

DUGT was purified from the conditioned media of *Drosophila* Kc 7E10 mass cell cultures by sequential $(NH_4)_2SO_4$ precipitation, velocity sucrose density gradient sedimentation, anion exchange and molecular sieve chromatography. Aliquots from the purification steps were electrophoresed on an SDS−7% polyacrylamide gel. Purified polyclonal anti-DUGT antibodies detected a single band of 170 kDa (Figure 1A and B). Examination of staged *Drosophila* embryo extracts with this antibody also detected a 170 kDa band only (Figure 1C). The native protein elutes from an analytical Superose 6 column (Pharmacia LKB Biotechnology) with M_r ~240 kDa relative to globular protein standards. However, DUGT is not globular as its hydrodynamic frictional ratio, f/f_0, is 1.5, corresponding to an equivalent hydrodynamic shape of an ~30 nm×3 nm rod. The approximate sedimentation coefficient ($s_{20,w}$) of DUGT is 6.9 S. The electrophoretic mobility of DUGT in SDS−PAGE is slightly decreased after reduction and increased after treatment with peptide:*N*-glycosidase F (unpublished data), indicating some intra-chain disulfide linkage and N-glycosylation. DUGT was electroblotted onto a PVDF membrane, and the 15 residue, NH_2-terminal sequence ESSQSYPITXLINAK was determined by microamino acid sequencing.

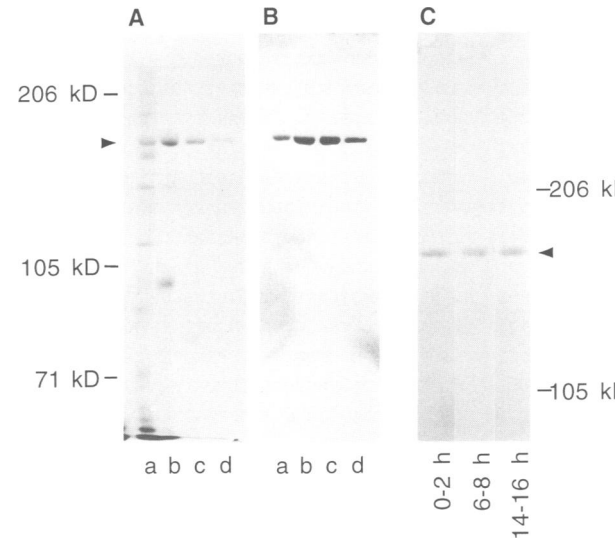

Fig. 1. Electrophoretic and Western blot analysis of DUGT. Aliquots of DUGT from successive purification steps were analyzed by SDS−7% PAGE and either Coomassie blue stained (**A**), or Western blotted and developed with affinity-purified antibodies to DUGT (**B**). The purification steps were (a) velocity sedimentation, (b) ion-exchange chromatography (Mono Q), (c) gel filtration chromatography (Superose 12) and (d) a second Mono Q column chromatography. (**C**) Extracts from *Drosophila* embryos of the indicated age ranges were resolved by a SDS−5% PAGE and a Western blot was developed with the above antibodies. Arrowheads indicate DUGT which migrates with an apparent M_r of ~170 kDa.

Cloning and sequence determination

Purified anti-DUGT serum was used to probe a cDNA expression library prepared from *Drosophila* Kc 7E10 cell RNA in the ZAP vector system (Nelson *et al.*, 1994). This yielded two overlapping clones that contained the 3′ end of the coding sequence and extended 1 and 2 kb, respectively, from the site of poly(A) addition. Subsequently DNA probes were used to isolate a 5317 bp cDNA clone containing the entire coding region of DUGT from a 4–8 h staged embryo cDNA plasmid library (Brown and Kafatos, 1988). An open reading frame of 4644 nucleotides codes for the 1548 amino acid residue sequence shown in Figure 2A. The cDNA extends 203 nucleotides 5′ of the initiator AUG and 469 nucleotides 3′ of the stop codon to a poly(A) addition signal AATAAA (Proudfoot and Brownlee, 1976) at nucleotide position 5295, 24 nucleotides upstream of the poly(A) tail. A signal sequence of 22 residues follows the initiator Met. The signal sequence cleavage site was predicted to be between Gly22 and Glu23 using the algorithm of von Heijne (1986). The NH_2-terminal sequence of the mature protein determined by microamino acid sequencing confirms this cleavage site. The molecular weight of the deduced protein sequence, without the signal peptide is 172 kDa. Apart from the signal peptide, DUGT lacks putative transmembrane sequences. There are three possible sites for N-glycosylation at residues 181, 266 and 864 (Figure 2A). Its GenBank accession number is U20554.

Searches of the Genbank, EMBL and SwissProt sequence databases show significant similarities with the seven sequences listed in Figure 2B. Two of these sequences, *Escherichia coli rfa*J (*E.coli* GT) and *Salmonella typhimurium rfa*I (Salty GT), are experimentally

A

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   1  MLRAVALCVSVVLIALYTPTSGESSQSYPITTLINAKWTQTPLYLEIAEYLADEQAGLFWDYVSGVTKLDTVLNEYDTESQQYNAALELVKSHVSSPQLP
 101  LLRLVVSMHSLTPRIQTHFQLAEELRSSGSCQSFTFAQVGSELACSFNELQKKLEVPLAKDSLDAPVVTYSFDHIFPGSENNTRTVVLYGDLGSSQFRTY
 201  HKLLEKEANAGRIRYILRHQLAKKDKRPVRLSGYGVELHLKSTEYKSQDDAPKPEAGSTSDEDLANESDVQGFDFKVLKQKHPTLKRALDQLRQRLLQGN
 301  DEIAQLKAWEFQDLGLQAAAAIAEIQGDETLQILQYTAHNFPMLARTLLAHKVTDGLRAEVKHNTEAFGRSLNVAPPDGALFINGLFFDADTMDLYSLIE
 401  TLRSEMRVLESLHSNNVRGSLASSLLALDLTASSKKEFAIDIRDTAVQWVNDIENDVQYRRWPSSVMDLLRPTFPGMLRNIRKNVFNLVLVVDALQPTAR
 501  SVIKLSESFVIHQAPIRLGLVFDARDANEDNLADYVAITCAYNYVSQKKDARAALSFLTDIYAAVGETKVVTKKDIVKQLTKEFTSLSFAKAEEFLEEDS
 601  TYDYGRELAAEFIQRLGFGDKEQPQALLNGVPMPSNVVTADSDFEEAIFTEIMTHTSNLQKAVYKGELTDNDVAIDYLMNQPHVMPRLNQRILSQEDVKY
 701  LDINGVAYKNLGNVGVLNRLSNRDMTATLMDNLKYFGGKKSTELIGRTSLQFLTIWVFADLETDQGRDLLTHALDYVQSGESVRVAFIPNTESSSASSRR
 801  NLNRLVWAAMQSLPPTQATEQVLKWLKKPKEKIEIPTQLEDILGSTELHLKMLRVYSQRVLGLNKSQRLVIGNGRLYGPLSSDESFDSADFALLARFSSL
 901  QYSDKVRQVLKESAQDVNEEFNSDTLLKLYASLLPRQTKTRFKLPTDLKTDHSVVKLPPKQEKLPHFDVAAVLDPASRAAQKLTPILILLRQVLNCQLNL
1001  YLIPVPQHSDMPVKNFYRYVVEPEVQFEANGGRSDGPLAKFSGLPANPLLTQQLQVPENWLVEAVRAVYDLDNIKLTDIGGPVHSEFDLEYLLLEGHCFD
1101  AASGAPPRGLQLVLGTQSQPTLVDTIVMANLGYFQLKANPGAWSLRLREGKSADIYAISHIEGTNTHHSAGSSEVQVLITSLRSHVVKLRVSKKPGMQQA
1201  ELLSDDNEQAAQSGMWNSIASSFGGGSANQAASDEDTETININFSVASGHLYERLLRIMMVSLLKHTKSPVKFWFLKNYLSPQFTDFLPHMASEYNFQYEL
1301  VQYKWPRWLHQQTEKQRTIWGYKILFLDVLFPLNVRKIIFVDADAIVRTDIKELYDMDLGGAPYAYTPFCDSRKEMEGFRFWKQGYWRSHLMGRRYHISA
1401  LYVVDLKRFRKIAAGDRLRGQYQALSQDPNSLSNLDQDLPNNMIHQVAIKSLPDDWLWCQTWCSDSNFKTAKVIDLCNNPQTKEAKLTAAQRIVPEWKDY
1501  DAELKTLMSRIEDHENSHSRDSAVDDSVDDSVEVTTVTPSHEPKHGEL*
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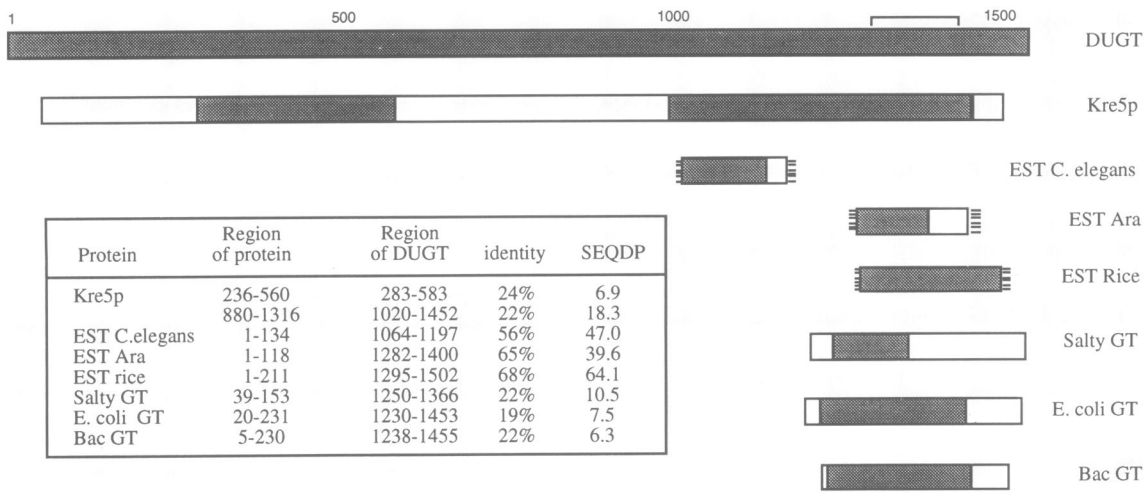
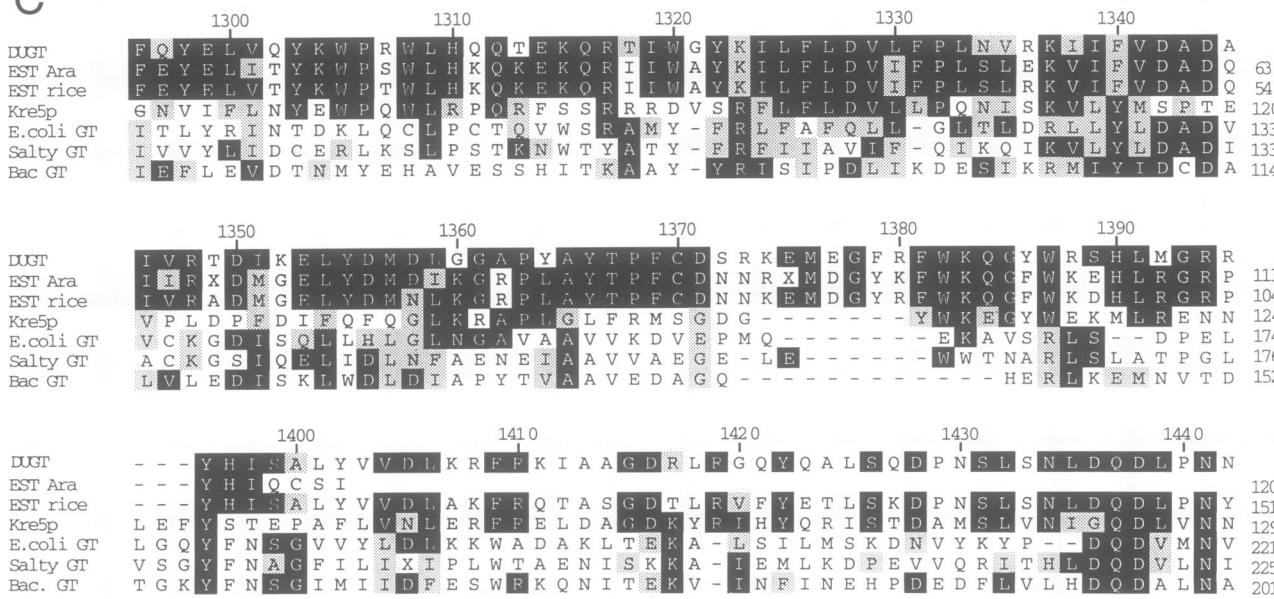
B



Protein	Region of protein	Region of DUGT	identity	SEQDP
Kre5p	236-560	283-583	24%	6.9
	880-1316	1020-1452	22%	18.3
EST C.elegans	1-134	1064-1197	56%	47.0
EST Ara	1-118	1282-1400	65%	39.6
EST rice	1-211	1295-1502	68%	64.1
Salty GT	39-153	1250-1366	22%	10.5
E. coli GT	20-231	1230-1453	19%	7.5
Bac GT	5-230	1238-1455	22%	6.3

C



determined glycosyltransferases and a third, *Bacillus subtillus* galactosyltransferase (Bac GT) is ascribed to a glycosyltransferase by homology. The *E.coli rfa*J gene product, lipopolysaccharide 1,2-glucosyltransferase and the *S.typhimurium rfa*I gene product, lipopolysaccharide 1,3-galactosyltransferase, use UDP-glucose and UDP-galactose respectively, to transfer distal sugars to a lipopolysaccharide core (Pradel *et al.*, 1992). Each glycosyltransferase consists of ~300 amino acid residues, of which substantial portions (114–225 residues) can be mapped to the same carboxyl region of DUGT. The most extensive similarity is shared with the *Saccharomyces cerevisiae Kre*5 gene product. Kre5 protein (Kre5p) is 1365 residues long and is homologous with DUGT in two separate regions: DUGT residues 283–583 and 1020–1452. Kre5p participates in the synthesis of the β1→6 glucan of yeast cell walls, although its specific function is not known (Meaden *et al.*, 1990). The sequences with the highest similarities to DUGT (up to 68% identity over 207 residues) are conceptual translations of expressed sequence tags (ESTs) from cDNA libraries of *Caenorhabditis elegans*, rice and *Arabidopsis*. Portions of these sequences are aligned with the COOH-terminal region of DUGT in Figure 2C. Many conserved and similar residues in invariant positions are evident. Three regions, a hydrophobic stretch (DUGT residues 1322–1330), (K/R)X(I/L)(Y/F)XDAD (residues 1337–1344) and DQDXXN (residues 1436–1441) are conserved among all sequences with slight variations. The latter region is noted to be conserved in both the *E.coli* and *S.typhimurium* analogs of *rfa*J and *rfa*I (Pradel *et al.*, 1992). Forced computer comparisons between currently listed sequences of hexosyl transfer enzymes in the International Enzyme Commission subset EC 2.4.1. and DUGT did not lead to any significant additional similarity alignments, though stretches of amino acid identity were found both in the COOH portion (residues 1000–1548) and in the preceding 1000 residues.

The COOH-terminal tetrapeptide sequence of DUGT (HGEL) and Kre5p (HDEL) are both variants of the canonical ER retrieval signal KDEL (Munro and Pelham, 1987). In *S.cerevisiae* HDEL acts as the ER retrieval signal (Pelham *et al.*, 1988). The HDEL COOH-terminus of Kre5p was demonstrated to be needed for proper function since a strain with this tetrapeptide deleted only partially rescued the kre5$^-$ mutant (Meaden *et al.*, 1990). To investigate the possibility that DUGT is a resident of the ER, its synthesis, location and secretion were examined.

Expression of DUGT

Northern analyses of RNA from *Drosophila* Kc cells and embryos revealed a single, ~5.5 kb message. Analysis of RNA from embryos collected at 2 h intervals indicated that DUGT message was present at low, but detectable levels in the earliest embryos, increasing at 6–8 h with a maximum at 10–12 h. Thereafter the DUGT mRNA level decreased and was not detected in 18–20 h embryos and first instar larvae, but was detected again at second instar to pupation (unpublished data). *In situ* hybridization and immunostaining of whole embryos showed that DUGT is generally expressed in cells of all embryonic tissues (Figure 3). More intense staining is seen in areas of higher cell density. DUGT was detected in oocytes of adult ovaries by antibody staining (unpublished data). Western blots of extracts of embryos made at 2 h intervals indicated approximately equivalent levels of extractable DUGT throughout development (Figure 1C). *In situ* hybridization mapped the gene for DUGT to chromosome locus 75D/E.

Subcellular localization of DUGT

Although DUGT was isolated from conditioned cell culture media, the majority of the protein appears to be retained intracellularly. Protein extracts of the cell layer and conditioned media were prepared from Kc 7E10 cells that had been cultured for 2, 4, 6 and 8 days. Western blots were prepared from equivalent amounts of each fraction and developed with purified anti-DUGT sera. As shown in Figure 4A, an almost undetectable level of DUGT is in the medium. In a separate Western blot analysis of serial dilutions of cell and media fractions, it was determined that ~95% of DUGT is retained in the cell fraction (unpublished data). Furthermore, DUGT was inaccessible to surface proteolysis as the level of DUGT in the cell extracts did not change after incubation of the intact Kc 7E10 cells with dispase (unpublished data), indicating an internal location. Immunostaining of *Drosophila* cultured cells (Figure 4B) and third instar salivary glands with anti-DUGT sera indicate a perinuclear, ER-like staining pattern similar to the pattern seen in these cells when stained with antibodies to the ER marker ribophorin I (Hortsch *et al.*, 1986). The presence of DUGT was demonstrated in microsomes prepared from Kc 7E10 cells both by Western blot analysis of extracts (Figure 4A) and by assay of glucosyltransferase activity (Table I).

Enzymatic activity of DUGT

To test whether DUGT is a soluble ER glucosyltransferase (Trombetta and Parodi, 1992), we assayed purified DUGT

Fig. 2. Amino acid sequence analyses of DUGT. (**A**) The conceptual translation of the 5317 nucleotide cDNA sequence starting at nucleotide 204. The 1548 amino acid sequence includes the signal sequence, shown in bold type, and the NH_2-terminal sequence determined from the isolated protein, shown underlined. The COOH-terminal four residues HGEL are in bold type. Potential sites of N-glycosylation are underlined. (**B**) The table lists regions of proteins which are similar to regions of DUGT with the indicated amino acid identity, and SEQDP relatedness scores (in standard deviation units). The proteins listed are Kre5p, *S.cerevisae* Kre5 protein; EST, expressed sequence tag of *C.elegans*, *Arabidopsis* and rice; Salty GT, *S.typhimurium rfa*I gene product, lipopolysaccharide 1,3-galactosyltransferase; *E.coli* GT, *E.coli rfa*J gene product, lipopolysaccharide 1,2-glucosyltransferase; Bac GT, gene ipa-12d of *B.subtilis* genomic region 325−333. The sequence of DUGT and the regions in other proteins that are potentially homologous are indicated by shaded boxes. Each box represents a complete protein sequence, except for the ESTs which have dashed borders, and is vertically aligned with the region of DUGT that corresponds to it. The bracket above the DUGT box indicates the region examined in (C). (**C**) The multiple sequence alignment of portions of proteins listed in (B) is shown, as determined by the GCG program PILEUP. The amino acid residues of DUGT are indicated by the top numbering. Identical residues, shared by three or more sequences at any given position, are boxed in black. Gray shading indicates residues that are similar (Dayhoff *et al.*, 1983).

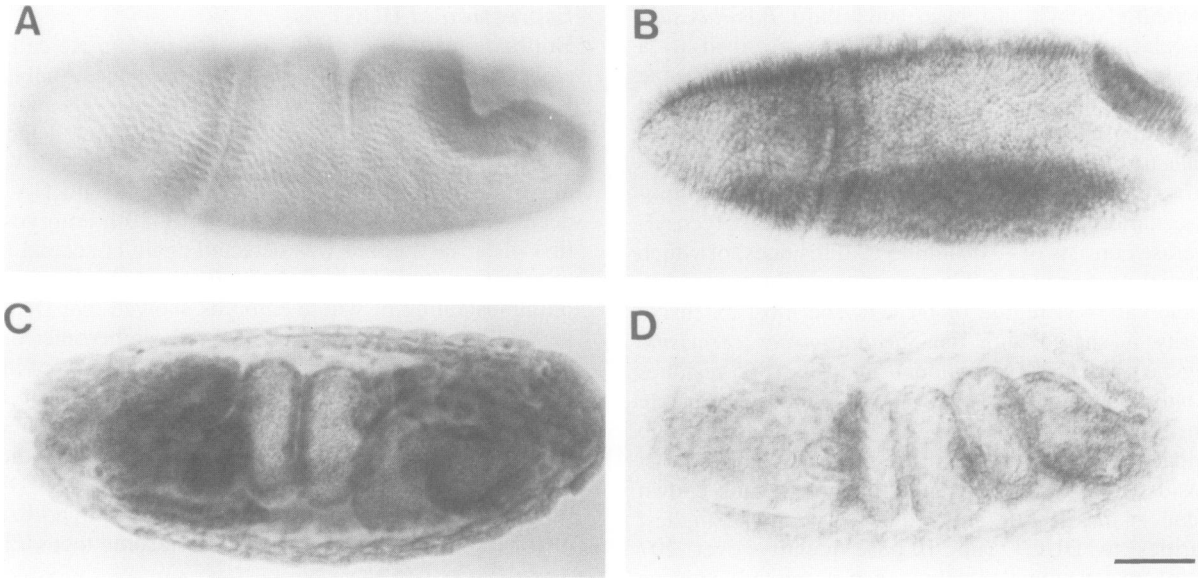


Fig. 3. Whole-mount immunostaining and *in situ* hybridization of *Drosophila* embryos. Stage 7 (**A** and **B**) and stage 16 (**C** and **D**) embryos were either immunostained with affinity-purified anti-DUGT antibodies (A and C) or hybridized with DUGT DNA probes (B and D). Embryos are shown with their anterior ends to the left. Bar indicates 100 μm.

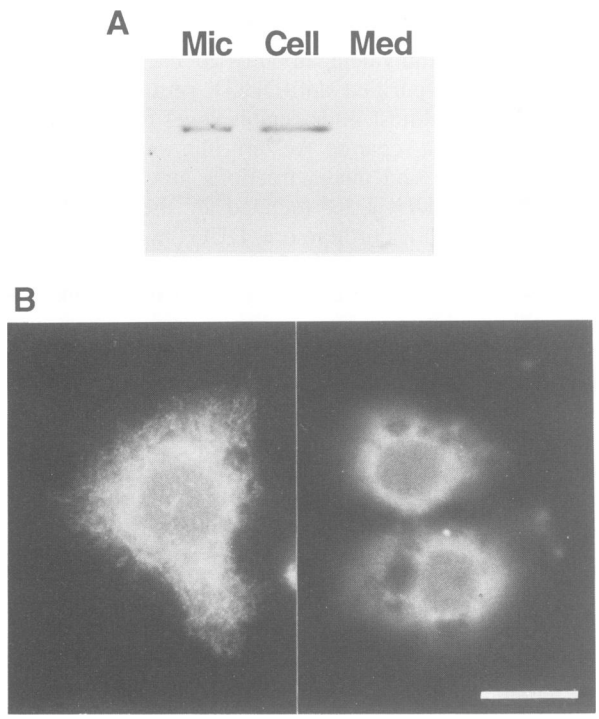


Fig. 4. Immunodetection of DUGT. (**A**) Western blot analysis of microsomal extract (Mic) and comparable amounts of Kc 7E10 cell extract (Cell) and conditioned culture medium (Med), developed with affinity purified anti-DUGT antibodies. (**B**) *Drosophila* S2 cells immunostained with affinity-purified anti-DUGT antibodies. Bar indicates 10 μm.

for UDP-Glc:glycoprotein glucosyltransferase activity; first on the standard test protein, bovine thyroglobulin and then on *Drosophila* laminin, peroxidasin and glutactin (Fessler *et al.*, 1994). DUGT catalyzes the transfer of radioactivity from UDP-[^{14}C]Glc to the denatured forms of these proteins with much greater activity than to the native forms. Table I displays the incorporation of

Table I. Glucosylating activity[a]

Substrate	c.p.m.
Thyroglobulin 200 μg	
native	238
urea-denatured[b]	9920
urea-denatured + 1 mM EDTA	193
urea-denatured + 1 mM EDTA +10 mM Ca^{2+}	10 721
urea-denatured + 1 mM EDTA +10 mM Mg^{2+}	568
urea-denatured + 1 mM EDTA +10 mM Mn^{2+}	7303
urea-denatured and Endo H pretreated	331
Laminin 15 μg	
native	167
heat-denatured	1054
heat-denatured; post-reaction Endo H treated	79
Peroxidasin 18 μg	
native	506
urea-denatured	3463
urea-denatured; post-reaction Endo H treated	160
Glutactin 10 μg	
native	1833
native; post-reaction Endo H treated	220
heat-denatured	4136
heat-denatured; post-reaction Endo H treated	606

Glucosylating activity of microsomes

Substrate	c.p.m.
Thyroglobulin 200 μg, urea-denatured	22 000
Thyroglobulin 200 μg, urea-denatured and Endo H treated	1090
Microsomes	2280
Microsomes, Endo H treated	160

[a]Purified DUGT was incubated with thyroglobulin, treated as indicated, for 1 h in the assay described in the methods section. Reactions with other substrates and with microsomes were incubated for 2.5 h.
[b]In a parallel experiment with DUGT that had been kept at 100°C for 10 min, 347 c.p.m. were incorporated.

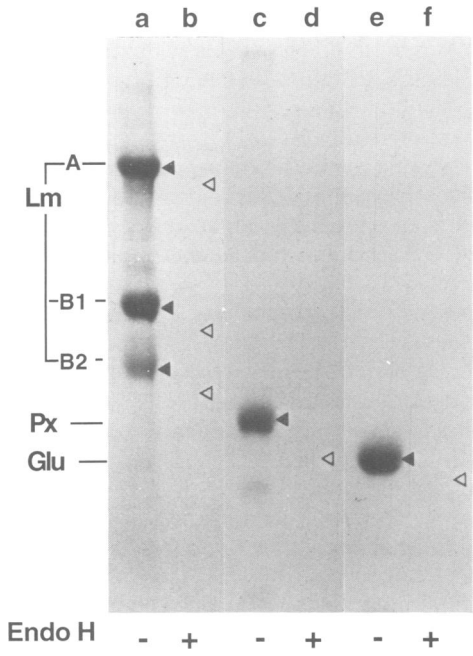


Fig. 5. Fluorogram of *Drosophila* extracellular matrix proteins. Denatured laminin, (lanes a and b), peroxidasin (lanes c and d) and glutactin (lanes e and f) were incubated with DUGT and UDP[^{14}C]Glc for 2 h. Samples b, d and f were then incubated with Endo H, and samples a, c and e with buffer. The products were analyzed by SDS−5% PAGE. The gels were first stained with Coomassie blue and the bands of the glycosylated (closed triangles) and de-glycosylated (open triangles) proteins were marked as indicated, then the fluorogram was developed. Endo H treatment resulted in increased electrophoretic mobility without protein degradation.

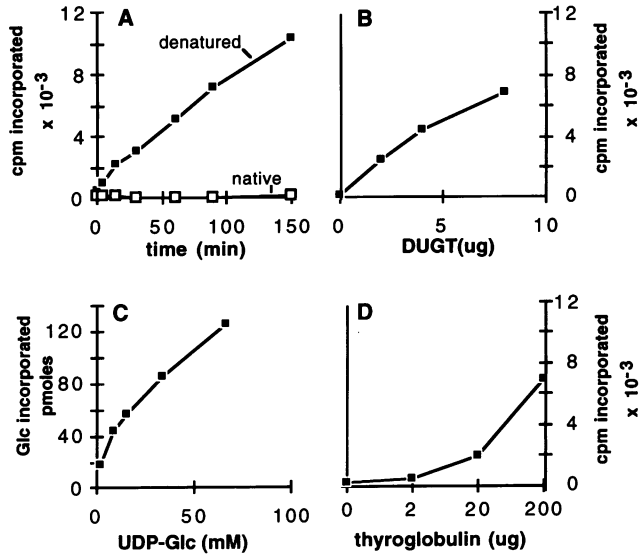


Fig. 6. Characterization of the glucosylating activity of DUGT. Glucosylation of 200 µg (560 pmol) denatured thyroglobulin by DUGT was assayed as described in methods. (**A**) shows incorporation of [^{14}C]Glc over time with denatured thyroglobulin (closed squares), and native thyroglobulin (open squares). (**B**) shows total incorporation of radioactivity with increasing amounts of DUGT, (**C**) shows the molar amounts of glucose incorporated with increasing UDP-Glc concentration during 4 h incubation. (**D**) illustrates incorporation of radioactivity with increasing mass of thyroglobulin substrate.

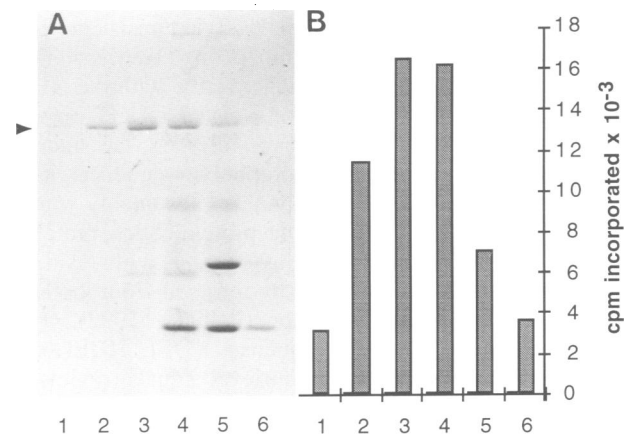


Fig. 7. Correlation of DUGT purification and glucosylating activity. Successive fractions of impure DUGT were eluted from a mono Q column and 45 µl samples were analyzed by SDS−7% PAGE. (**A**) shows the gel stained with Coomassie blue. Arrowhead indicates the position of DUGT. (**B**) shows the glucosyltransferase activity of 5 µl of fractions corresponding to (A) with 200 µg urea-denatured thyroglobulin in 1 h.

radiolabelled glucose into different substrates by the action of DUGT or *Drosophila* microsomes. The enzymatic activity is stimulated by both Ca^{2+} and Mn^{2+}, but not Mg^{2+} ions and is destroyed by heating DUGT. As demonstrated with the rat UDP-Glc:glycoprotein glucosyltransferase (Trombetta *et al.*, 1989), pretreatment of thyroglobulin with endoglycosidase H blocks acceptor activity. Conversely, Endo H treatment of the substrates after glucosylation removes the radiolabel, consistent with the supposition that the transferred [^{14}C]glucose becomes covalently attached to high mannose oligosaccharides. This is verified by the fluorogram of glucosylated laminin, peroxidasin, and glutactin electrophoresed on an SDS−5% polyacrylamide gel (Figure 5). There was no evidence of proteolysis of these proteins by Endo H, as bands of intact polypeptides were seen in all lanes when the same gel was stained with Coomassie blue. As shown in Figure 6, glucose incorporation increased with incubation time, concentration of both UDP-Glc and polypeptide substrate, and with the total DUGT in the reaction mixture. Figure 6A shows that native thryoglobulin does not act as a substrate even at longer incubation times.

To verify that the observed glucosyltransferase activity is entirely due to the protein that was isolated and sequenced, and not due to some contaminant, glucosyltransferase activity was assayed throughout an isolation of DUGT. The enzymatic activity was found in conditioned media of Kc 7E10 cell cultures and was subsequently associated with the 170 kDa band on SDS−PAGE. Figure 7 shows an example of parallel electrophoretic and enzymatic analyses of successive fractions of impure DUGT eluted from a Mono Q ion exchange column; the enzymatic activity was unrelated to residual contaminants.

Discussion

We have purified DUGT to homogeneity, and obtained the cDNA sequence of this novel *Drosophila* glucosyltransferase which transfers glucose from UDP-Glc to denatured glycoproteins. The correlation of glucosylating

activity with the purification of the 170 kDa protein confirms that this polypeptide is the enzyme. The identity of this peptide's NH_2-terminal amino acid sequence with that of its cDNA deduced protein sequence confirms the cloning of the glucosyltransferase. Futhermore, this protein is a glucosyltransferase of *Drosophila* microsomes as judged by enzymatic and immunological criteria.

Characterization of DUGT isolated from *Drosophila* cell culture shows that it is the functional homolog of the UDP-Glc:glycoprotein glucosyltransferase isolated from the microsomal fraction of rat liver (Trombetta and Parodi, 1992). The 170 kDa DUGT is of a similar size to the 150 kDa rat liver glucosyltransferase, both on reduced SDS−PAGE and by native gel filtration. Both proteins glucosylate denatured, but not native, bovine thyroglobulin and other substrates that have an endoglycosidase H-sensitive oligosaccharide acceptor. Both proteins require UDP-Glc and Ca^{2+} for activity, (although Mn^{2+} cations can also stimulate the activity in *Drosophila*).

Even though DUGT was isolated from long-term cell culture media, immunodetection of most of DUGT in extracts of the cells, rather than in the medium, suggests that it is primarily an intracellular protein. The immunostaining patterns of cells with anti-DUGT antibodies are consistent with an ER-localization of the enzyme. The fine meshwork-like, cytoplasmic staining of DUGT is primarily perinuclear (Figure 4), and consecutive optical sections, obtained by confocal microscopy, showed that the strongest staining enveloped nuclei in a pattern similar to the staining for ribophorin I (unpublished data).

The amino acid sequence of DUGT is consistent with an ER location of the protein. Comparison of the translation of the cDNA sequence open reading frame with the NH_2-terminal amino acid sequence of isolated DUGT proves that a signal peptide was cleaved off. As the mature DUGT lacks any extended hydrophobic sequence, it is not a transmembrane protein, and is presumably translocated into the ER as a soluble protein. Such proteins may be retained in the ER by association with resident membrane bound complexes or by retrieval from the *cis*-Golgi by a retrograde receptor (Pelham, 1992). The COOH-terminal tetrapeptide sequences KDEL, HDEL and some variations thereof, act as retrieval signals (reviewed by Pelham, 1990). Egasyn and several other mammalian ER carboxyesterases terminate with HXEL, where X = I, T, V (Zhen *et al.*, 1993). The COOH-terminal sequence of DUGT, HGEL, could be a new variant of the retrieval signal for ER proteins, although this remains to be tested. Presumably the appearance of DUGT in conditioned culture media represents an imperfect retrieval mechanism, that may be exacerbated in mass roller cultures of the highly productive Kc 7E10 cells. The secretion of KDEL-containing, soluble ER resident proteins, such as protein disulfide isomerase (PDI), from highly productive cells has been described. Immunogold electron microscopy showed that PDI was limited to the lumen of the ER in rat hepatocytes, but was present throughout the secretory pathway in exocrine pancreatic cells (Akagi *et al.*, 1988).

The occurrence of DUGT protein and its mRNA among all types of embryonic cells indicates that it is synthesized and utilized by most cells. The presence of DUGT mRNA at the earliest embryonic times, and the staining of oocytes for DUGT (data not shown), indicate maternal contributions that are supplemented with the start of zygotic transcription. The stages which show a decrease in DUGT mRNA, limited to late embryos and first instar larvae, coincide with a period of *Drosophila* development when there is relatively little growth; a time when the need for protein synthesis machinery would possibly be diminished. The approximately constant level of protein extracted from embryos of all stages is consistent with a stable DUGT serving a fundamental function throughout development.

The protein sequence analysis indicates that this is a novel glucosyltransferase with no clear homologies to eukaryotic glucosyltransferases listed in the databases. Preliminary sequence data of the rat UDP-Glc:glycoprotein glucosyltransferase shows a sequence of 100 amino acids with 68% identity with DUGT (Dr A.J.Parodi, personal communication). This provides independent, additional confirmation for our identification of this *Drosophila* enzyme. Furthermore, this stretch of homology occurs within the same region of DUGT as its similarity with the bacterial glycosyltransferases. Although comparisons of DUGT with bacterial proteins (Figure 2B) show only 19–22% amino acid identities, all alignments are at meaningful levels of significance. Since the alignment covers the majority of the bacterial glycosyltransferase sequences, the bacterial catalytic site of sugar transfer is likely to be contained within the region of homology. A portion of this region of homology is likely to contribute to the glucosyltransferase catalytic site of DUGT. The sequence alignment highlights several stretches of similar and identical residues in the COOH-terminal region of DUGT shared by all the proteins.

The ability to discriminate between native and denatured substrate proteins is an inherent property of DUGT that was equally observed in crude material and in the most highly purified preparations. This suggests that DUGT does not need accessory proteins to recognize a wide variety of denatured protein substrates. How DUGT does this is not known. The domain structures of the glutactin, laminin and peroxidasin substrates are quite different as judged by electron microscopy, amino acid sequence and chain composition. Each of the different chains of the disulfide-linked, heterotrimeric laminin molecule were glucosylated, to different degrees, by DUGT. The most likely common denominator of such widely different proteins is exposure of some normally hidden hydrophobic regions by denaturation. The simplest mechanism of DUGT recognition would involve binding to a hydrophobic patch of the unfolded proteins. There are several difficulties with a 'hydrophobic patch recognition' model. First, the glucosyltransferase functions in the presence of the detergent Triton X-100, which perturbs some hydrophobic interactions. Secondly, as found by Sousa and co-workers (1992) and our unpublished observations, the presence of the de-glycosylated form of a denatured substrate protein inhibits glucosylation of a substrate that has the mannose acceptor. The recognition of a hydrophobic patch by DUGT is likely to be linked to concurrent recognition of some part of the substrate's N-linked oligosaccharides. Otherwise DUGT's enzymatic activity would be inhibited by random encounters with partly folded proteins that lack any N-linked sugars. Interestingly, glucose transfer by the rat liver enzyme to

pronase-derived glycopeptides proceeds more slowly, by two orders of magnitude, than to glycoprotein substrates (Sousa *et al.*, 1992). This implies some interactions between the protein recognition and the catalytic transfer sites (review: Baenziger, 1994).

The hydrodynamic evidence (frictional ratio f/f_o) indicates that DUGT differs distinctly from a compact, globular shape. This allows models of DUGT in which the sites for catalytic glycosylation and for recognition of malfolded protein might be formed by different regions of its polypeptide chain, yet still be brought near to each other by interactions with the substrate. Interestingly, both the *Drosophila* and rat glucosyltransferases are fairly large proteins that could provide extended surfaces for protein−protein interactions. The yeast protein Kre5p is also of a similar size, and the two regions of homology with DUGT in the NH_2- and COOH-terminal ends could represent these two important functional domains. The strong relationship of DUGT with the yeast Kre5 protein supports the idea that Kre5p may be a glucosyltransferase involved in early steps of yeast β1→6 glucan biosynthesis (H.Bussey, personal communication). The high sequence identities seen with the ESTs of *Arabidopsis*, rice and *C.elegans* cDNAs suggest that these sequences represent proteins that have conserved function and may be the DUGT homologues of these organisms.

The *Drosophila* extracellular matrix proteins that acted as excellent substrates for DUGT *in vitro*, had probably encountered DUGT during their assembly within the ER of the Kc 7E10 cells. Glucosylated high mannose dolichol-linked oligosaccharide precursors have been identified in *Drosophila*, though the N-linked glycosylation pathway has not been studied extensively (Parker *et al.*, 1991). Most, or all, of the N-linked oligosaccharides of the secreted proteins that we used are of the high mannose type, as digestion with either endoglycosidase H or F cleaved the individual proteins to a similar size. This is consistent with the observations of N-linked oligosaccharide processing in other insect cell cultures (O'Reilly *et al.*, 1992). The *in vitro* glucosylation of these extracellular matrix proteins indicates that the terminal glucose residues had been trimmed from the mature proteins.

The ability of DUGT to distinguish denatured glycoproteins from native substrates, as evidenced by *in vitro* glucosylation, makes this enzyme a logical selection as a monitor protein to identify glycoproteins that need assistance in folding from chaperones. Recent reports show that several newly made glycoproteins become transiently attached to the membrane-bound chaperone calnexin within the ER (Ou *et al.*, 1993). Furthermore, mutations or amino acid substitutions that are known to inhibit folding of some glycoproteins increase this association with calnexin (Hammond *et al.*, 1994). The assembly of some glycopolypeptides into higher complexes is also associated with calnexin (reviewed in Bergeron *et al.*, 1994). Evidence indicates that calnexin selectively binds monoglucosylated N-linked glycoproteins (Hammond *et al.*, 1994). The following model of the processing of some glycoproteins was proposed, in which calnexin and UDP-Glc:glycoprotein glucosyltransferase have central, complementary roles (Hammond and Helenius, 1993; Hammond *et al.*, 1994). A newly made glycopeptide becomes monoglucosylated, either by trimming of $Glc_3Man_9GlcNAc_2$ to $Glc_1Man_9GlcNAc_2$, or by the action of glucosyltransferase on $Man_9GlcNAc_2$. Calnexin will bind the monoglucosylated glycoprotein that is in need of assistance of folding and thus restrain it from leaving the ER. The refolded protein dissociates from calnexin and the Glc is split off by ER glucosidase II. After dissociation from calnexin, the refolded protein encounters glucosyltransferase which tests its state of folding. If the enzyme detects a denatured state it glucosylates the substrate and thereby returns the protein to a further round of association with calnexin. Only correctly folded proteins evade glucosylation and leave the ER. Persistently malfolded chains would be re-glucosylated several times, and would eventually be degraded by other, unknown mechanisms.

The full range of DUGT as a monitor of protein folding is unknown. It might only check a limited repertoire of folding motifs. However, the presence of DUGT in a wide variety of embryonic tissues suggests that DUGT-controlled folding/export mechanisms are not confined to the synthesis of specialized extracellular matrix proteins and are widely used. Experiments to test the proposed role of DUGT in the folding and assembly of *Drosophila* proteins are in progress.

Materials and methods

Isolation and characterization of DUGT

Roller bottle cultures of *Drosophila* Kc 7E10 cells were grown in D22 medium, without serum. Conditioned cell culture media were clarified, protease inhibitors were added, proteins were precipitated at 45% $(NH_4)_2SO_4$ saturation and partially separated by velocity sedimentation as described (Fessler *et al.*, 1994). DUGT sedimented to fractions 9–12 from the tube bottom (out of 21 fractions) in a 5–20% sucrose gradient with a 60% sucrose pad, centrifuged in a Beckman SW41 rotor at 39 000 r.p.m. and 4°C for 19 h. Pooled fractions were dialyzed into 300 mM sucrose, 30 mM Tris−HCl, pH 7.25, 10 mM EDTA, 0.1% Triton X-100, 0.02% sodium azide and applied to a FPLC Mono Q anion exchange column (Pharmacia LKB Biotechnology). The column was developed with a 0–0.5 M NaCl gradient in the same buffer and DUGT eluted at ~0.123 M NaCl. DUGT was monitored as a 170 kDa band on SDS−PAGE. DUGT was further purified by gel filtration on an FPLC Superose 12 column (Pharmacia LKB Biotechnology) in 300 mM sucrose, 150 mM NaCl, 50 mM Tris−HCl, pH 7.5, 0.05% Triton X-100, 5 mM EDTA, 0.02% sodium azide. If needed, residual contaminants were removed by repeating the Mono Q chromatographic step. The purified DUGT was electrophoresed on an SDS−5% polyacrylamide gel, electroblotted onto PVDF membrane (Bio-Rad), stained with Coomassie Blue and rinsed. NH_2-terminal sequence was determined directly from the band corresponding to DUGT using an automated amino acid sequencer, model 477 (Applied Biosystems Inc.) equipped with on-line phenylthiohydantoin amino acid analyzer and model 900 data analysis system. Previously described methods (Fogerty *et al.*, 1994) were used to determine the sedimentation coefficient $s_{20,w}$ and to calculate the frictional ratio, f/f_o, from $s_{20,w}$ and the sequence-derived M_r = 172 kDa. The hydrodynamic parameters and the derived, hydrodynamically equivalent molecular shape were relatively insensitive to the values assumed for the partial specific volume (0.73 ml/g) and hydration (0.1) .

Antibody preparation and immunostaining

A rat was immunized with non-reduced DUGT band cut out of an SDS−5% polyacrylamide gel. The antisera were further purified to remove traces of contaminating glutactin and laminin reactive antibodies by passage through columns of these proteins linked to activated Sepharose (Pharmacia LKB Biotechnology). Affinity-purified antibodies were prepared by incubating nitrocellulose-bound, reduced DUGT with the polyclonal antisera (Robinson *et al.*, 1988; Fessler *et al.*, 1994). Western blots were performed as described (Harlow and Lane, 1988). Extracts of staged, dechorionated embryos were made by homogenization in 10 vol of 2% SDS, 150 mM NaCl, 50 mM Tris−HCl pH 7.5, 20 mM EDTA; heated to 100°C for 5 min; sonicated; dithiothreitol (DTT) was

added to 10 mM, the extracts were heated again to 100°C for 5 min and centrifuged. Protein concentrations were determined using the Bio-Rad protein assay. Aliquots of equivalent amounts were electrophoresed and Western blots were developed. To assay the amount of DUGT in the cell and media fractions, Kc 7E10 cells from confluent cultures in T75 flasks were dislodged, washed and similarly extracted. The proteins of the corresponding conditioned culture media were precipitated at 50% $(NH_4)_2SO_4$ saturation in the presence of protease inhibitors, resuspended and dialyzed into 150 mM NaCl, 50 mM Tris−HCl pH 7.5, 10 mM EDTA, and clarified. Equivalent fractions were electrophoresed on an SDS−5% polyacrylamide gel, under reducing conditions, electroblotted onto nitrocellulose, stained with purified antibodies and alkaline phosphatase-conjugated second antibody. Whole embryos were immunostained as described (Olson *et al.*, 1990; Fogerty *et al.*, 1994). *Drosophila* S2 cells expressing PS2 integrin (Gotwals *et al.*, 1994) were placed on a coverslip coated with vitronectin (10 μg/ml) and allowed to spread for 2 h. The cells were fixed and stained with affinity-purified antibody to DUGT and anti-rat FITC-conjugated IgG, or with antibody to ribophorin I (Hortsch *et al.*, 1986) and anti-mouse FITC-conjugated IgG.

cDNA library screening and DNA sequencing

A portion (5×10^6 p.f.u.) of a *Drosophila* Kc 7E10 cell cDNA λZAP expression library (Nelson *et al.*, 1994) was plated on *E.coli* strain YS1 and screened with anti-DUGT sera according to standard methods (Sambrook *et al.*, 1989). Two overlapping immunopositive clones were isolated; both of which contained poly(A) tails. A fragment (residues 3125–3795) of the clone that extended farthest in the 5′ direction was used to screen a Southern blot prepared from 18 subpools (1.4×10^4 c.f.u.) each of the pNB40 12–24 h *Drosophila* embryo cDNA library (Brown and Kafatos, 1988) as described (Fogerty *et al.*, 1994). A 3.5 kb clone was isolated from one subpool. The 5′ most fragment of the larger clone (residues 2026–2613) was used to screen the pNB40 4–8 h *Drosophila* embryo library in the same manner. A 5317 bp clone containing the entire coding region was isolated.

Restriction fragments (no larger than 2 kb) of the longest clone were subcloned into pBS (Stratagene) or pMOB for use in transposon (Tn) facilitated sequencing (Strathmann *et al.*, 1991). The PCR reactions of appropriate Tn transformants were diluted up to 50-fold with water, concentrated in a centricon-100 (Amicon) to remove the primers, and directly sequenced by automated sequencing. Additional sequence was obtained by double-stranded sequencing of selected restriction fragment subclones and exonuclease III/mung bean nuclease nested deletion subclones (Stratagene) using the Sequenase sequencing kit (US Biochemicals). The DNA sequence data of both strands were assembled and analyzed using the GCG sequence analysis package. Databases GenBank (release 84), EMBL (release 39) and SwissProt (release 29) were searched with Blast (NCIB) and the GCG programs TFasta and Fasta. Sequence matches were identified that had a Fasta optimized score greater than 100, which also had increased from the initial (intn) score. Pairwise matches were further analyzed with the GCG program Bestfit and Los Alamos programs SEQHP and SEQDP. Accession numbers of proteins homologous with DUGT are Kre5p: M33556; EST *C.elegans*: M75929; EST *Arabidopsis*: T23006; EST rice: combination of D24144 and D24933; *S.typhimurium rfa*I gene product, lipopolysaccharide 1,3-galactosyltransferase, Salty GT: P19816; *E.coli rfa*J gene product, lipopolysaccharide 1,2-glucosyltransferase, *E.coli* GT: P27129; gene ipa-12d of *B.subtilis* genomic region 325− 333, Bac GT: X73124.

Northern analyses and in situ hybridization to chromosomes and embryos

Total RNA was isolated from staged Oregon R embryos, larvae, and Kc 7E10 cells and Northern analyses were done as described (Kusche-Gullberg *et al.*, 1992) with a [^{32}P]RNA probe transcribed from a plasmid which contains the 3′-terminal 1 kb of DUGT cDNA. *In situ* hybridization to *Drosophila* polytene chromosomes were performed according to the method of Langer-Safer *et al.* (1982) using biotin-UTP nick-translated transcripts of the same DUGT-cDNA plasmid. Random primed digoxigenin-labelled DNA probes corresponding to DUGT nucleotide sequences 1513–1951 and 2961–3459 were used for *in situ* hybridization to whole embryos (Tautz and Pfeile, 1989).

Glucosyltransferase assay

Glucosyltransferase activity was assayed essentially as described in Trombetta and Parodi (1992). Five μl of the enzyme fraction was incubated in a total volume of 50 μl 10 mM $CaCl_2$, 300 μM deoxynojirimycin (Boehringer Mannheim), 2.5 μM UDP-[^{14}C]glucose, 300 mCi/mmol (Dupont), 5 mM Tris−HCl pH 8.0 with the appropriate substrate at 26°C. The reaction products were precipitated on ice for 30 min by the addition of 1 ml 10% trichloroacetic acid, then poured onto filter paper and washed with at least 2 ml each of 10% trichloroacetic acid, 95% ethanol and acetone. The filters were dried and incorporated radioactivity was counted.

Urea-denatured and Endo H (New England BioLabs) treated bovine thyroglobulin (Sigma) were prepared as described in Trombetta *et al.* (1989). *Drosophila* laminin, peroxidasin and glutactin were isolated from the conditioned media of Kc cell culture conditioned media as described (Fessler *et al.*, 1994). Laminin and glutactin were denatured by heating to 65°C for 15 min then quick cooling on ice. Peroxidasin was denatured by sequential dialysis to (i) 8 M urea, 20 mM Tris, pH 7.5 at room temperature for 2 h; (ii) the buffer in step 1 with 10 mM DTT added for 6 h; (iii) 6 M urea, 20 mM Tris pH 7.5, 20 mM *N*-ethylmaleimide for 0.5 h; (iv) 50 mM Tris pH 7.5, 0.15 M NaCl at 0°C for 3 h. Post-reaction Endo H treatment was according to manufacturer's instructions.

Drosophila microsomal fractions were prepared from Kc 7E10 cells suspended (1 g/10 ml) in 10 mM HEPES buffer pH 7.5, with 2 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml each of aprotinin, leupeptin and pepstatin at 0° C for 10 min, then homogenized in a Potter−Elvehjem homogenizer. To the homogenate were added: sucrose to 0.25 M, triethanolamine, pH 7.5 to 50 mM, potassium acetate to 50 mM and magnesium acetate to 6 mM. The homogenate was centrifuged for 10 min at 1900 *g* to remove whole cells and nuclei. The supernatant was then centrifuged for 10 min at 7000 *g*, then again at 105 000 *g* for 1 h. The final pellet was suspended in 1 ml/g 0.25 M sucrose, 50 mM triethanolamine, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 3 μg/ml each of aprotinin, leupeptin and pepstatin with a Dounce homogenizer.

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