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The *Drosophila GMII* gene encodes a Golgi α-mannosidase II

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SUMMARY

In this paper we show the organisation of the *Drosophila* gene encoding a Golgi α-mannosidase II. We demonstrate that it encodes a functional homologue of the mouse Golgi α-mannosidase II. The *Drosophila* and mouse cDNA sequences translate into amino acid sequences which show 41% identity and 61% similarity. Expression of the *Drosophila* GMII sequence in CHOP cells produces an enzyme which has mannosidase activity and is inhibited by swainsonine and by $CuSO_4$. In cultured *Drosophila* cells and in *Drosophila* embryos, antibodies raised against a C-terminal peptide localise this product mainly to the Golgi apparatus as identified by cryo-immuno electron microscopy studies and by antibodies raised against known mammalian Golgi proteins. We discuss these results in terms of the possible use of dGMII as a *Drosophila* Golgi marker.

Key words: α-Mannosidase II, Golgi apparatus, Genomic sequence, Glycosyl transferase, N-glycosylation pathway, *Drosophila* S2 cell, *Drosophila* embryos, Indirect immunofluorescence, Cryo-immuno electron microscopy

INTRODUCTION

The function of N-linked oligosaccharides has been investigated by studying the consequence of blocking the process of glycosylation at different stages. One such approach makes use of inhibitors such as 1-deoxyjirimycin or swainsonine (see review by Fuhrmann et al., 1985) to interfere with the glycosylation pathway. There are two objections to this approach. Firstly, there is no way of anticipating what other aspects of the biology of the organism might be disturbed by the inhibitors. Secondly, in theory at least, the inhibitors may themselves be specific for the N-glycosylation pathway but they may also be metabolised into compounds that affect other pathways.

An alternative approach, which avoids these problems, is to look for spontaneous or induced disturbance of genes coding for enzymes in the N-linked glycosylation pathway and to study the effects of those disturbances. For example gene knock-out experiments in the mouse have shown that mutations of *N*-acetylglucosaminyl transferase I are lethal (Metzler et al., 1994; Ioffe and Stanley, 1994) and that the removal of mannosidase II results in dyserythropoietic anaemia (Chui et al., 1997).

Drosophila melanogaster is an obvious choice for the genetic approach since it is feasible, in this organism, to carry out genetic manipulations some of which are difficult or impossible in higher eukaryotes. Examples include mutation and overexpression, ectopic gene expression and generation of mosaic flies by mitotic recombination.

From the work of Williams et al. (1991), Parker et al. (1991) and Kerscher et al. (1995), it seems likely that the N-linked glycosylation pathway in *Drosophila* is essentially the same as that in mammals, at least up to the synthesis of GlcNac2Man5. Kerscher et al. (1995) reported a mutation that lacked Golgi mannosidase I activity and had but slight phenotypic disturbances, and we showed (Roberts et al., 1998) that there was likely to be an alternative pathway to circumvent the block caused by mutations of Golgi mannosidase I. A similar analysis in *Drosophila* of mutations affecting genes encoding other enzymes in the N-linked glycosylation pathway would reveal either the extent to which there are alternative pathways or the consequence of blocking the pathway at a particular step.

An ideal candidate for the genetic approach is *Drosophila* Golgi α-mannosidase II (dGMII), which is one of the early enzymes involved in complex oligosaccharides synthesis. The cloning of the cDNA and the mapping of the putative gene (*dGMII*) for this enzyme was reported by Foster et al. (1995). This gene encodes a protein with considerable homology to the mouse α-mannosidase II (GlcNAc transferase I-dependent α1,3 [α1,6] mannosidase, EC 3.2.1.114) (mGMII) (41% identity and 61% similarity). It is a typical type II transmembrane protein like mGMII, although the two

transmembrane domains show no sequence similarity. However, overall sequence identity/similarity is not sufficient to confirm that this gene codes for an enzyme with Golgi α-mannosidase II (GMII) activity in *Drosophila*.

Here, we report on the cloning of the genomic sequence and on its characterisation. We demonstrate that this protein has α-mannosidase activity which is strongly inhibited by swainsonine, a feature of GMII. Finally, we further demonstrate that this gene codes for a protein which is localised in the Golgi apparatus using immunofluorescence and cryo-immuno electron microscopy on *Drosophila* S2 cells and embryos. By these definitions dGMII is the *Drosophila* homologue of mammalian Golgi α-mannosidase II.

MATERIALS AND METHODS

Expression of *Drosophila* and mouse GMII in CHOP cells

Both *Drosophila* and mouse GMII sequences were cloned into the pProtA expression vector (Sanchez-Lopez et al., 1988) for expression in CHOP cells. CHOP cells are CHO cells stably expressing polyoma large T-antigen (Hefferman and Dennis, 1991). pProtA allows the expression of protein fused to a 248 amino acid fragment of Protein A under the control of the SV40 early gene promoter. An amino terminal signal sequence from rat stromelysin causes the expressed protein to be secreted to the medium.

The dGMII insert consists of a 3267 bp *Nru*I/*Sna*BI fragment isolated from the cDNA clone pNB40-GMII (Foster et al., 1995). This was inserted into Klenow-filled *Eco*RI cut pProtA+1 (pProtA with a 1 bp insertion in front of the *Eco*RI site (K. Moremen, unpublished). Insert orientation was determined by colony PCR and DNA for transfection was prepared using the PEG precipitation procedure (Sambrook et al., 1989). The resulting Protein A-GMII fusion contains residues 75-1108 of dGMII. The cytosolic and transmembrane domains are absent, as is a portion of the 'stalk' connecting the transmembrane domain to the catalytic domain.

The mGMII was prepared from the cDNA (Moremen and Robbins, 1991). Briefly, the 5′ end was amplified by PCR using an upstream primer containing an *Eco*RI restriction site and a Factor Xa recognition sequence (Ile-Glu-Gly-Arg), and a downstream primer which anneals to a sequence about 80 bp downstream of a unique *Xma*I site. The construct was created by a triple ligation with *Eco*RI-*Xma*I digested PCR product, a 3179 bp *Xma*I-*Eco*RI fragment from the cDNA and *Eco*RI digested, dephosphorylated pProtA. The resulting secreted protein contains a Protein A domain, a Factor Xa cleavage site and mannosidase starting at amino acid Arg-35, 8 amino acids after the end of the predicted transmembrane domain. Controls consisted of CHOP cells transfected with the pProtA vector alone.

CHOP cells were transiently transfected using lipofectamine reagent (Life Technologies) as recommended by the manufacturer. After 72 hours of cell culture, secreted recombinant enzyme was isolated from the cell culture medium using IgG-Sepharose Fast Flow beads (Pharmacia Biotech) to bind to the Protein A fusions. 5 μl of a 50% bead slurry, 2.5 μl of 2 M Tris-HCl (pH 8.0), and 5 μl of 10% Tween-20 were added per ml of culture medium. Following incubation on a rocking platform at 4°C for 20 hours, beads were collected by centrifugation, washed twice in TST (50 mM Tris-HCl, pH 8, 150 mM NaCl and 0.05% Tween-20), and re-suspended in 1/100 of the original culture volume of TST.

GMII activity measurements

Unless otherwise indicated (see legend for Fig. 2), measurements of α-mannosidase activity were carried out in 40 mM MES buffer, pH 5.75, and 5 mM *p*-nitrophenyl α-D-mannopyranoside (PNP-Man, Sigma) using a reaction volume of 50 μl in 96-well microtitre plates (Falcon 3911, Becton-Dickinson). All components, including inhibitors or salts, were added to the reaction mixture before the addition of the enzyme bound to IgG-Sepharose beads. The plates were sealed with Parafilm to prevent evaporation and incubated at 37°C for various times (typically 1-2 hour). The reaction was stopped by the addition of 50 μl of 500 mM sodium carbonate and the absorbance measured at 405 nm using a plate reader. Blanks contained Sepharose beads without substrate. All reactions and controls were carried out in triplicate and the results averaged.

Western blotting of pProtA fusion proteins

IgG-Sepharose beads containing bound dGMII were boiled for 2 minutes in SDS-PAGE sample buffer. After centrifugation, the supernatant was loaded onto 8% acrylamide SDS-PAGE gels. Blotting to nitrocellulose was carried out in Tris-glycine buffer in the absence of methanol. The membrane was blocked with 2% bovine serum albumin in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20). Fusion protein was detected on the basis of the tight binding of Protein A to rabbit IgG. A non-specific antiserum was diluted 1:500 in TBS-T containing 2% BSA and allowed to react with the blot for 1 hour at room temperature. After washing, horseradish peroxidase-conjugated goat anti-rabbit IgG was incubated with the blot for 1 hour at room temperature. The blot was developed using diaminobenzidine staining.

Raising antisera against dGMII

In order to recognise the dGMII protein and to localise the protein in cells, we raised antibodies against a synthetic peptide consisting of the carboxy-terminal 14 amino acids (CPMETAAYVSSHSS). This was coupled to chicken ovalbumin and the product was used to prepare antibodies commercially (Serotec) in rabbits. The rabbit bleeds were tested by an ELISA plate assay, using the peptide coupled to catalase as the target antigen.

Characterisation of the anti-dGMII serum

The anti-dGMII serum was characterised further by analysing extracts of wild-type embryos and embryos from the P4.24.3 strain using the western blot technique. The P4.24.3 transformant has dGMII cDNA placed under the control of GAL4 UAS (DBR unpublished results) (Brandt and Perrimon, 1993). This strain is crossed to a transformed strain expressing GAL4 under the control of an ubiquitous enhancer (these strains, Mz827, Mz449 and Mz759/TM3 were the gift of Joachim Urban). The resulting embryos overexpress GMII driven by GAL4 UAS.

Embryos were collected overnight, de-chorionated and homogenised in 200 μl of homogenisation buffer (15 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 10 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 0.25 M sucrose) using a plastic Dounce homogeniser. An aliquot was immediately mixed with an equal volume of 2× SDS sample buffer, boiled for 5 minutes and frozen. A cocktail of protease inhibitors (Boerhinger) were added to the remainder of the homogenate which was centrifuged for 3 minutes at 1000 rpm to remove cell debris. The supernatant was centrifuged on a 25 μl cushion of 2.3 M sucrose at 100,000 rpm for 1 hour in a TLA.100 Beckman labtop centrifuge to separate the membrane fraction from the cytosol. The membrane fraction was recovered and mixed with an equal volume of 2× SDS sample buffer, boiled, and frozen. The cytosol was precipitated with methanol/chloroform, the pellet was mixed with SDS sample buffer, boiled and frozen.

dGMII was also expressed in *Spodopteran* cells using a Baculovirus expression vector (D. B. Roberts, unpublished results). A sample of transfected and non-transfected cells, as well as a sample of the supernatant, were mixed with SDS sample buffer, boiled and frozen. After loading in a 10% acrylamide gel and blotting, the nitrocellulose filter was incubated with the anti-dGMII serum and visualised using the ECL system (Amersham). Autoradiographs were scanned and processed with Adobe Photoshop.

Indirect immunofluorescence microscopy

Drosophila S2 cells were cultured on polylysine coated glass coverslips in Schneider medium (Sigma) supplemented with 12.5% heat inactivated fetal bovine serum, 1 mM glutamine and penicillin/streptomycin. Near confluent cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature; rinsed with 50 mM ammonium chloride; and fixed with −20°C methanol for 6 minutes. Immunofluorescence single labelling was carried out by the method of Nilsson et al. (1993). Briefly: fixed cell-coated coverslips were rinsed in 0.2% fish skin gelatin in PBS (PBSG); incubated with primary antibodies diluted in PSBG for 30 minutes at room temperature; rinsed three times with PBSG; incubated with secondary anti-rabbit antibodies coupled to FITC; rinsed successively in PBSG, PBS and water; mounted on Vectashield (Vector) and examined using a confocal laser scanning microscope (Leica). Pictures from confocal microscopy were processed with Adobe Photoshop.

Double labelling experiments were carried out as follows (all incubations were carried out for 30 minutes at room temperature): fixed cell-coated coverslips were incubated with anti-dGMII serum; rinsed; incubated successively with anti-rabbit antibody coupled to FITC, 1% normal rabbit serum in PBSG, and a second primary antibody. This was followed either by a biotinylated anti-rabbit antibody and then streptavidin coupled to Texas Red, or by an anti-rabbit antibody coupled to HRP and then an anti-HRP antibody coupled to FITC. Since both primary antibodies were raised in rabbits, a control experiment was carried out omitting the second primary antibody, and only low background was observed. Antibodies against various Golgi markers were used: anti-dGMII, anti-rat Gos28 (gift from Thomas Söllner); anti-rat syntaxin 5 (gift from Graham Warren), and anti-delta subunit for the AP-3 coat system (gift from Margaret Robinson). Pictures from confocal microscopy were processed with Adobe Photoshop.

Wild-type embryos were collected for 1 hour and aged for 6 hours; de-chorionated with 5% sodium hypochlorite; fixed with 4% paraformaldehyde/heptane for 20 minutes at room temperature; and incubated with methanol at −20°C overnight. The cells were re-hydrated by successive 5 minutes washing in 95%, 70%, 50% and 30% methanol in PBS, PBS alone and PBS-0.1% Tween-20 (PBST). After washing in PBST supplemented by 1% BSA (Sigma) for 10 minutes, they were incubated overnight at 4°C with anti-dGMII serum diluted to an appropriate concentration with PSBT containing 0.1% BSA. After extensive washing in PBST (at least 3 washes over a period of 2 hours), they were incubated with an anti-rabbit secondary antibody coupled to FITC for 1 hour at room temperature. They were rinsed in PBST, PBS and water and mounted as before. Pictures from confocal microscopy were processed with Adobe Photoshop.

Cryo-immunolocalisation and quantitation

Near confluent *Drosophila* S2 cells growing in a 10 cm dish were fixed in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, for 3 hours at room temperature. Cells were recovered; washed three times in PBS and PBS supplemented with 50 mM ammonium chloride; and processed for cryo-immunoEM as previously described (Rabouille et al., 1995). Ultrathin cryosections were cut on a Leica S4 Ultracut, labelled using the anti-dGMII serum followed by 10 nm Protein A gold (Cell biology laboratory, Utrecht School of Medicine), stained with uranyl acetate, embedded in methyl cellulose and viewed with a CM10 Philips transmission electron microscope.

In order to quantify labelling, at least 15 different views were photographed and analysed. The views included a section of the nucleus in order to facilitate orientation and position within the cells. Boundaries of the nucleus, endosomes and endoplasmic reticulum (ER) (comprising the nuclear envelope) were drawn by following the most outer membrane. The Golgi apparatus was drawn as defined in Rabouille et al. (1995) and comprised the stack of cisternae and the membrane networks abutting it. The 'point-hit' method was applied using photographs at a magnification (mag) of 52.5 K, and a grid with a spacing (d) of 1 cm, except for the ER where the spacing was 0.5 cm. The number of points, P (grid intersections), falling in each organelle was recorded. The surface area (S, μm^2) is $P \times d^2/mag^2$. It follows that the labelling density (LD) is the number of gold particles (Ng) associated with one organelle divided by the surface area of this organelle LD=Ng/S. The specific LD for each organelle was calculated by subtracting the background, which is defined as the labelling density over the nucleus (LD nucleus) The relative distribution of gold particles was expressed as a percentage of the total number of specific gold particles (Rabouille et al., 1999).

Wild-type embryos were collected for 3 hours and aged for 15 hours; de-chorionated with 5% sodium hypochlorite; fixed with a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4/heptane for 30 minutes at room temperature and hand-peeled (de-vitellinised) according to Van den Heuvel et al. (1989). Briefly, the fixative layer was removed, the embryos and the heptane phase were placed on a plastic Petri dish and the excess of heptane thoroughly removed. The embryos, that now stick to the dish, were covered by PBS and gently displaced out of the vitelline membrane using with a sharp needle. About 15-30 embryos were collected and post fixed in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.2M phosphate buffer, pH 7.4, for 2.5 hours at room temperature. They were rinsed and processed as described above for the S2 cells. Ultrathin cryosections were cut and labelled with the anti dGMII antiserum as described above followed by 10 nm Protein A gold.

ΔdGMII-HA$_3$ and NAGT1-myc construct

The sequence encoding the first 105 amino acids of dGMII (from the start codon +4 downstream) including the cytoplasmic domain, the transmembrane domain and the stalk region (just before the catalytic domain) was amplified by PCR from the full cDNA sequence. The primers were designed to create two restriction sites *Eco*RI at the 5′ end and *Xho*I at the 3′ end. This truncated dGMII (ΔdGMII, 315 bp) was cloned into pRD67 in frame with the HA3 cassette. The ΔdGMII-HA$_3$ sequence (400 bp) was cut using *Eco*RI and *Bgl*II and subcloned into pRMHa-3 cut with *Eco*RI and *Bam*HI. The insertion was checked by digestion the plasmid with *Eco*RI/*Sal*I which gives a 4 kb fragment.

The cDNA sequence of the human NAGT1 tagged with the myc epitope (NAGT1-myc, 1.5 kb) (Nilsson et al., 1993) was cut from pCmuiV vector using *Bam*HI and subcloned into pMK33 cut with *Bam*HI. The orientation was checked by digesting with *Sac*II/*Spe*I which gives a 1 kb fragment.

Transient transfection of *Drosophila* S2 cells was carried out using calcium phosphate precipitation directly on confluent cells growing on polylysine coated coverslips. After 48 hours of transfection, cells transfected with either ΔdGMII-HA$_3$ or NAGT1-myc were fixed with 4% paraformaldehyde only, and double labelled with anti-HA (Babco) and anti-myc (9E10) monoclonal antibody respectively, followed by a donkey anti-mouse antibody coupled to Texas Red; and with the anti-dGMII serum followed by a donkey anti-rabbit coupled to FITC. Pictures from confocal microscopy were processed with Adobe Photoshop.

RESULTS

Cloning the *dGMII* gene

We cloned the *dGMII* genomic sequence in order to transform the gene into flies. We have shown (Lockyer, 1995) that this transformed sequence is expressed together with the native sequence. The transformed sequence can now be used to rescue and define mutations of *dGMII*.

dGMII cDNA was mapped to 85D14-18 on the polytene chromosome by in situ hybridisation (Foster et al., 1995). A

series of *Drosophila* P1 clones have been prepared, under the auspices of the Berkeley *Drosophila* Genome Project, and many have been mapped to the polytene chromosome (Hartl et al., 1994). We received three clones (DS05016, DS01769 and DS06620) which mapped to the 85D region. Restriction digests of these clones were probed. The *dGMII* cDNA probe only hybridised to a 6 kb *Eco*RI fragment from DS05016 (mapped to 85D11-17). This was sub-cloned into the *Eco*RI site of pUC18 (P1E-E).

A comparison of the PCR products using the same pairs of primers but with either *dGMII* cDNA or P1E-E DNA as template showed, in several instances, that the latter gave a larger PCR fragment which would be explained by the presence of introns. We sequenced the 6 kb insert to investigate the organisation of the *dGMII* gene.

The P1E-E sub-clone was sequenced, on both strands, by the Sanger dideoxy sequencing method using primers derived from the cDNA sequence and where necessary from new genomic sequence. Fig. 1A shows the overall structure of *dGMII*. The intron splice sites are shown in Fig. 1B. The complete sequence has been deposited at GenEMBL accession number AJ 132715.

There were two significant base differences between the genomic and cDNA sequences (base 365 and 3663 from start of translation in cDNA sequence), one resulted in a glutamate-lysine polymorphism, and was confirmed by running the cDNA and genomic DNA sequences in adjacent tracks (data not shown). The second difference resulted in another glutamate-lysine polymorphism and generated a *Hin*dIII site in the cDNA. Polymorphism for this *Hin*dIII site had previously been detected in an analysis of inbred *Drosophila* stocks (data not shown).

Analysis of the upstream sequence does not reveal any sequence that has been implicated in transcription control. There is no obvious TATA box (see Bucher, 1990), no Initiator sequence (see Cherbas and Cherbas, 1993) and no CAAT box, although this is not common in *Drosophila* (Arkhipova, 1995). Nevertheless this 6 kb fragment has all the information for the expression of GMII since a construct containing a shortened 3′UTR sequence transformed into *Drosophila* is co-expressed with the native gene (Lockyer, 1995).

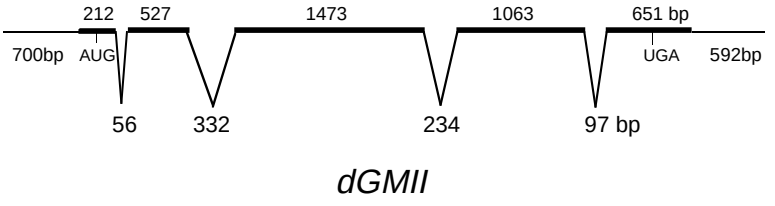
Expression of active α-mannosidase in CHOP cells

dGMII and mGMII were expressed as Protein A fusion proteins in transiently transfected CHOP cells. The pProtA vector used for the expression encodes a rat stromelysin signal sequence which causes the expressed fusion proteins to be secreted into the medium. The Protein A moiety allows easy purification of the expressed proteins by its tight affinity for IgG-Sepharose.

The presence of the two proteins on the beads was tested by western blot (Fig. 2A). The migration position of the bands corresponds to the expected size of the fusion proteins which is 147 kDa for the *Drosophila* enzyme and 156 kDa for the mouse enzyme.

The presence of α-mannosidase activity was determined by the ability to cleave the artificial substrate PNP-Man. No α-mannosidase activity could be purified from the medium of cells transfected with the empty pProtA vector. However, when either *dGMII* or *mGMII* sequence was present α-mannosidase

A



dGMII

B

5' consensus sequence										
	-3	-2	-1	1	2	3	4	5	6	7
All species	A/C	A	G	G	T	A/G	A	G	T	A/T
Drosophila	A/C	A	G	G	T	A/G	A	G	T	A/T
GMII intron I	A	A	G	G	T	A	A	A	T	A
GMII intron II	G	T	C	G	T	A	A	G	C	A
GMII intron III	A	G	A	G	T	G	A	G	T	A
GMII intron IV	A	A	G	G	T	G	A	G	C	T

3' consensus sequence									
	-7	-6	-5	-4	-3	-2	-1	1	2
All species	C/T	C/T	C/T		C	A	G	G	
Drosophila	C/T	T	T		C	A	G	A/G	T
GMII intron I	T	T	A	G	C	A	G	C	C
GMII intron II	T	T	T	A	C	A	G	C	A
GMII intron III	C	T	T	A	C	A	G	G	C
GMII intron IV	T	C	G	T	C	A	G	C	T

Fig. 1. The structure and sequence of the *Drosophila* Golgi α-mannosidase II gene. (A) The general structure of the gene with the cDNA sequence is shown as a thick line. (B) The dGMII 5′ and 3′ intron splice site sequences are shown together with the consensus splice site sequences for all species and for *Drosophila melanogaster*. The complete sequence of the P1E-E clone has been assigned EMBL/GenBank accession number AJ32715.

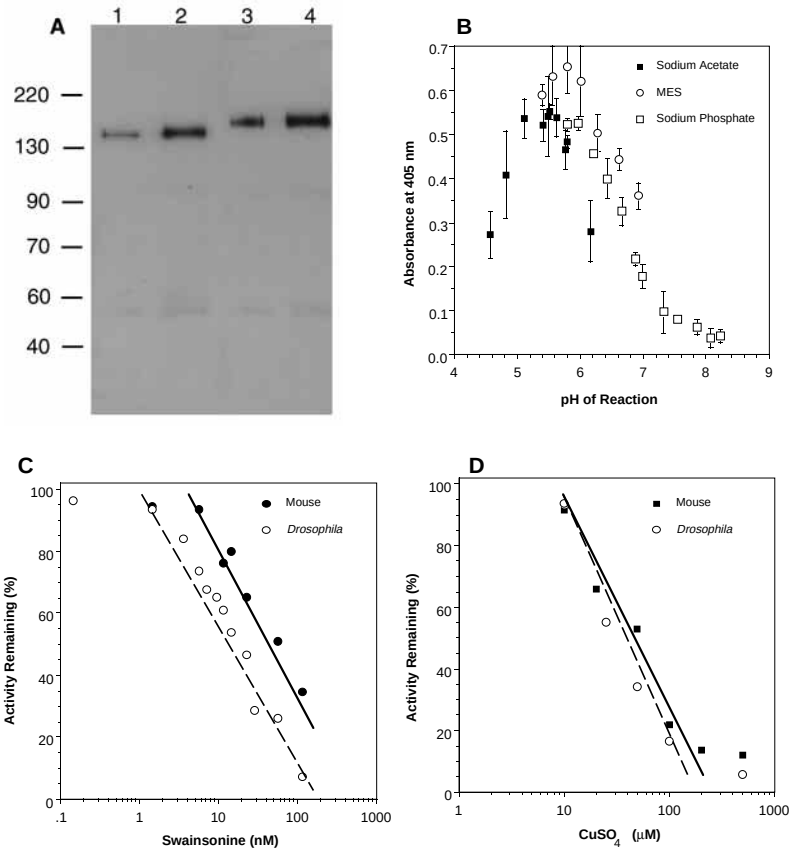


Fig. 2. α-Mannosidase activity of *Drosophila* GMII and mouse GMII. (A) The production of the two mannosidases was confirmed by western blotting. CHOP cells were transiently transfected with pProtA-dGMII or pProtA-mouse GMII and the secreted proteins were isolated from the medium with IgG-Sepharose. The size of the secreted products was analysed by SDS-PAGE on 8% acrylamide gels, blotted on nitrocellulose filters and visualised as outlined in Materials and Methods. dGMII (lanes 1 and 2) and mouse GMII (lanes 3 and 4). The loading in lanes 1 and 3 contained 1.7 μl of bead suspension, and 5 μl in lanes 2 and 4. The apparent molecular mass of the markers (Benchmark prestained protein ladder, Life Technologies) is indicated and ranged from 220 to 40 kDa. (B) Enzyme activity, using PNP-Man as the substrate, was measured as indicated in the text and a pH profile of the mannosidase activity established. The final pH of the reaction mixture with all components added as measured and reported in the x-axis. (C) Inhibition of both mannosidases with swainsonine was measured. All reaction components including inhibitor were added before the addition of mannosidase bound to IgG-Sepharose. Linear regression analysis on the linear part of the plot resulted in an IC_{50} value of 16.9 nM for dGMII and 55.1 nM for mouse GMII. (D) The inhibition of *Drosophila* and mouse mannosidase by copper sulphate was determined under the same conditions used for the swainsonine inhibition assay (regression analysis resulted in an IC_{50} of 33.3 μM for dGMII and 44 μM for mouse GMII. In C and D, the 100% activity is similar for both dGMII and mGMII.

activity was observed bound to the beads. Due to difficulties eluting the enzymes from the beads in an active form we performed all the enzymatic characterisation with bead-bound enzymes. Measurement of α-mannosidase activity of the dGMII protein at temperatures ranging from 16 to 42°C showed a linear increase (slope of 2.8, data not shown). The activity was subsequentely measured at 37°C.

For comparison of the insect enzyme with mammalian homologues, studies with Protein A-mGMII fusions bound to IgG-Sepharose were carried out in parallel. The mouse construct is similar to the *Drosophila* construct with the cytosolic and transmembrane domains being absent, but a larger portion of the stalk is present which contains 2 potential glycosylation sites (Moremen and Robbins, 1991). However, a deletion mutant (starting at residue 126), which eliminates the majority of the stalk including these glycosylation sites, retained greater than 75% of its activity (D.A.K, unpublished results).

The pH profile of dGMII was determined between pH 4.5 and 8.5 in a range of buffers (Fig. 2B) which included sodium acetate, MES, sodium phosphate and Tris. Optimal activity was obtained in MES pH 5.7. The pH profile is similar to that seen for other GMIIs (Moremen and Robbins, 1991; Ren et al., 1997; Kaushal et al., 1990) and is distinct from that of lysosomal mannosidases which have pH optima close to 4.5.

dGMII was strongly inhibited by swainsonine (Fig. 2C) with 50% inhibition of the enzymatic activity (IC_{50}) seen between 12-20 nM. This value is similar to that reported for GMII isolated from *Spodopteran* cells (Ren et al., 1997). Under the same reaction conditions, the mGMII had an IC_{50} of 60 nM. This value is lower than previously reported for the rat enzyme (Tulsiani et al., 1985) but this may reflect differences in the assay conditions.

Finally, we tested dGMII for metal ion dependency. $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnSO_4$, $NiSO_4$, $BaCl_2$, LiCl, or NaCl at concentrations up to 10 mM had no effect on the activity (data not shown). EDTA also had no effect on activity up to 10 mM (data not shown). In contrast, $CuSO_4$ was a strong inhibitor of dGMII with an IC_{50} of about 25 μM (Fig. 2D). $CuSO_4$ sensitivity of insect cell GMII has previously been reported (Ren et al., 1997; Altmann and Marz, 1995), as has inhibition of the enzyme from Mung bean Golgi apparatus (Kaushal et al., 1990). We were unable to find previous reports of mGMII inhibition by $CuSO_4$, but found it was indeed sensitive under our reaction conditions (Fig. 2D) in a range similar to that for the other GMII enzymes.

Characterization of the anti-dGMII serum

We raised antibodies against a 14 amino acid peptide corresponding to the C terminus of dGMII. Western blots of *Drosophila* embryo extracts show that the anti-dGMII serum recognised a band at about 115 kDa that corresponds to the calculated molecular mass of dGMII (Fig. 3). Increasing amounts of wild-type embryo extracts (5 to 250 μg protein) were loaded on the gel and the intensity of the band increased accordingly (data not shown). In a second experiment, equal amounts of total protein from wild-type embryos and from embryos from a *Drosophila* transformant overexpressing dGMII, p4.24.3, were loaded on acrylamide gels. The intensity of the band detected with the anti-dGMII increased 4 to 5 times

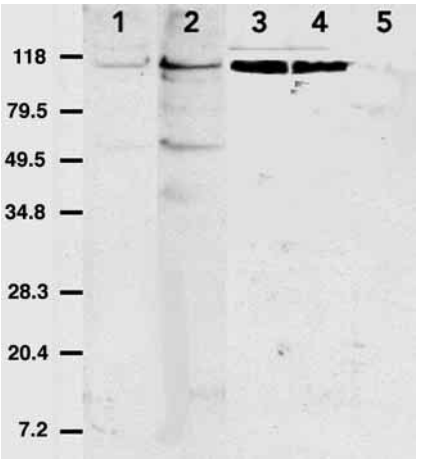


Fig. 3. Characterisation of the dGMII antiserum by western blotting. Different types of biological materials were used to test the specificity of the anti-dGMII antiserum. *Drosophila* wild-type embryos homogenate (lane 1, 88 μg protein loaded), P4.24.3 overexpressing mutant for dGMII embryo homogenate (lane 2, 68 μg), *Drosophila* wild-type embryo homogenate in the presence of proteases inhibitors and concentrated 5 times (lane 3, 440 μg), membrane fraction corresponding to the sample in lane 3 (lane 4, 112 μg), cytosol corresponding to the sample in lane 3 (lane 5, 403 μg). The molecular mass (in kDa) of the markers (prestained, from Bio-Rad) is indicated.

which corresponds to an expected overexpression (Fig. 3, lanes 1 and 2).

A second faint band at around 60 kDa was detected in both the wild-type and p4.24.3 homogenates. In view of the increased intensity of this band in p4.24.3 it was likely to be related to dGMII, e.g. a degradation product, such a product has been reported for the rat GMII (Slusarewicz et al., 1994). To test this, wild-type embryos were homogenised in the presence of protease inhibitors and concentrated 10 times. The intensity of the band detected with the dGMII antibody increased but the contaminant disappeared (Fig. 3, lane 3).

The membrane fraction (Fig. 3, lane 4) and the cytosolic fraction (Fig. 3, lane 5) of wild-type embryo homogenates were also examined using the anti-dGMII serum. It detected proteins in the membrane fraction, but none was detected in the cytosolic fraction.

The anti-dGMII serum was checked further by probing an extract of *Spodopteran* cells infected with Baculovirus carrying a dGMII construct. The same amount of protein from infected and non-infected *Spodopteran* cell extracts were analysed. No band was decorated in the non-infected extract (data not shown). We also obtained a positive results when extracts of *Drosophila* S2 cells were probed with anti-dGMII serum (data not shown).

Cellular distribution of dGMII

In *Drosophila* S2 cells

The anti-dGMII serum was first used to localise dGMII by immunofluorescence microscopy on S2 cells (Fig. 4A and B). The single labelling experiment revealed a punctate pattern around the label-free nucleus, consisting of several spots scattered in the cytoplasm, consistent with the Golgi apparatus as observed in plant cells (Henderson et al., 1994) or in yeast (Chappell and Warren, 1989). This *Drosophila* pattern was quite different from the pattern observed with mammalian cells (Burke et al., 1982; Nilsson et al., 1993) in which the Golgi apparatus is observed as a ribbon-like single copy organelle capping the nucleus. As a control, the anti-dGMII antiserum was preincubated with the peptide against which the antiserum was raised. This resulted in a considerable decrease of the signal (data not shown).

In order to confirm that the *Drosophila* pattern is due to staining of the Golgi apparatus we used antibodies raised against established mammalian Golgi markers, syntaxin 5 (Hui et al., 1997, data not shown), Gos28 (Nagahama et al., 1996; Fig. 4C) and delta subunit from AP-3 coat complex (Simpson et al., 1997, Fig. 4D) in single labelling experiments on *Drosophila* S2 cells. The patterns obtained were very similar to that obtained with dGMII anti-serum. Several spots were scattered in the cytoplasm around the nucleus.

In a double labelling experiment using the antiserum to dGMII and antibodies to the mammalian Golgi markers mentioned above, co-localisation of dGMII and the delta subunit was almost complete (Fig. 5A) as it was for Gos28 (Fig. 5B) and syntaxin 5 (Fig. 5C) although areas of non overlap were observed.

Drosophila S2 cells were fixed, processed for cryo-immunoelectron microscopy, sectioned and labelled using the anti-dGMII serum which was detected with Protein A coupled to 10 nm gold particles (Fig. 6). The Golgi apparatus was clearly identifiable consisting of stacks of flattened cisternae surrounded by tubular/vesicular membranes that might represent the cis- and trans-Golgi networks. The stacks, though, appeared smaller in diameter than in mammalian cells and were not seemingly linked into a large ribbon. Rather, they appeared discrete in the cytoplasm and it was common to observe 2 or 4 per cell section. Gold particles corresponding to dGMII decorated the Golgi apparatus of S2 cells but some

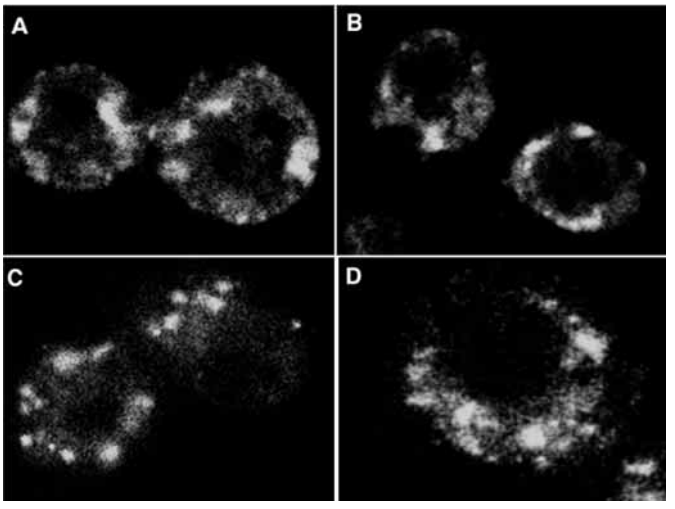


Fig. 4. Intracellular distribution of dGMII in *Drosophila* S2 cell line. *Drosophila* S2 cells were fixed, permeabilised and singly labelled with the antiserum to (A,B) dGMII, and polyclonal antibodies to (C) Gos28 and to (D) the delta subunit of the AP-3 coat. FITC-coupled secondary antibodies were used to detect primaries. Pictures were generated using a confocal microscope and processed using Adobe Photoshop. They represent a single confocal section.

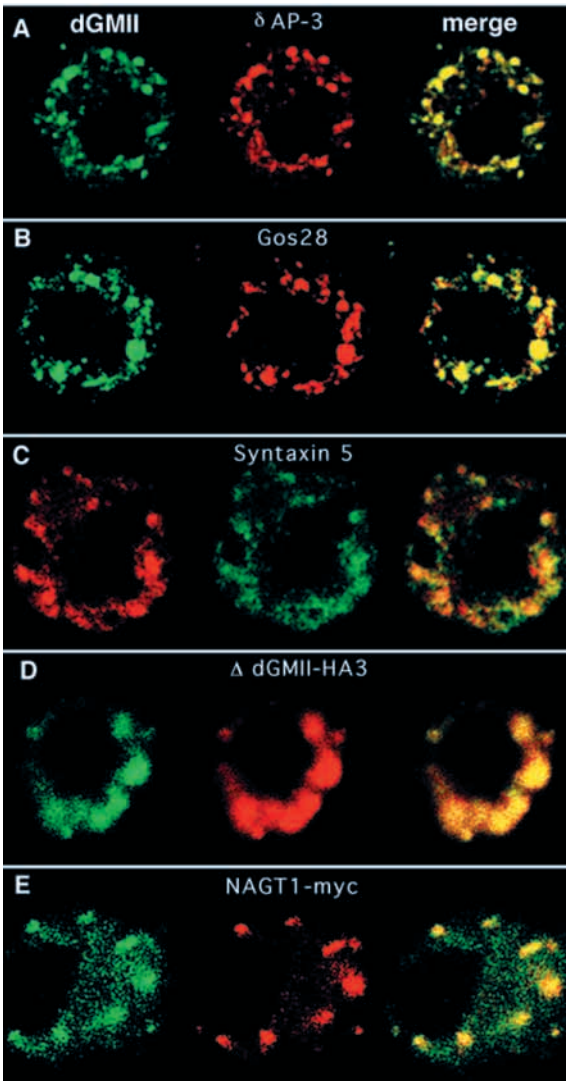


Fig. 5. dGMII colocalises with Golgi markers. S2 cells were double labelled using the antiserum to dGMII and a series of Golgi markers, (A) the delta subunit of AP-3, (B) Gos28 and (C) syntaxin 5. The anti-dGMII antiserum was used as the first primary antibody and was detected using anti-rabbit antibodies coupled to FITC (A,B) or Texas Red (C). The second primary antibody (against the mammalian Golgi markers) was either detected using a biotinylated goat anti-rabbit antibody followed by a streptavidin coupled to Texas Red (A,B), or using a donkey anti-rabbit antibody coupled to HRP followed by a anti-HRP antibody coupled to FITC (C). The control was performed omitting the second primary antibody. A low background was observed. S2 cells transiently transfected with (D) dGMII-HA3 or (E) NAGT1-myc were double labelled using anti-HA and anti-myc, respectively, followed by donkey anti-mouse antibody coupled to Texas Red and the anti-dGMII antiserum followed by donkey anti-rabbit antibody coupled to FITC. The punctate areas where both markers co-localise are shown in yellow whereas the green or red areas correspond to no colocalisation.

were also found associated with the ER (endoplasmic reticulum) cisternae and endosomes. In order to investigate whether the labelling was specific, the labelling density (LD: number of gold particles per surface area of organelle) was calculated for the nucleus (background), the endosomes, the ER and the Golgi apparatus comprising the stack of cisternae plus the two networks abutting it. The boundaries were drawn as defined by Rabouille et al. (1995). The background (in the nucleus) was found to be 0.72 gold particle/μm^2. The LD in the Golgi apparatus was 6.3, in endosomes 1.1 and in the ER cisternae 2.5. This gives a relative distribution of gold particles of 61%±10% in the Golgi apparatus, 10%±3% in endosomes, 29%±7% in the ER. The plasma membrane was devoid of labelling. The labelling of dGMII within the Golgi apparatus was not all associated with the stack. In fact, most of the labelling was at the edge of the stack and in the associated networks of membranes.

In *Drosophila* embryos

To visualise the immunofluorescence pattern of dGMII in *Drosophila* embryos, 6 hour old embryos were labelled using the anti-dGMII serum. These displayed a pattern similar to that observed in *Drosophila* S2 cells (Fig. 7A). Around the clearly visible nucleus a punctate pattern (1.5 spots per nucleus on average) was observed that might correspond to the Golgi apparatus in vivo (Fig. 7B). To confirm that the immunofluorescence pattern observed with the dGMII antiserum correspond to labelling of the Golgi apparatus, we performed a single immunolabelling on ultrathin cryosections of 18 hours old embryos. At that age, the Golgi apparatus displayed a very typical architecture of stacked cisternae and was clearly labelled by gold particles corresponding to dGMII (Fig. 8). Occasionally, the ER cisternae were also labelled by gold particles as in S2 cells.

A truncated form of dGMII and mammalian resident Golgi enzymes are also Golgi markers in S2 cells

In order to understand more about the localisation of dGMII and mammalian Golgi enzymes in the Golgi apparatus of *Drosophila* S2 cells and tissues, two types of experiments were carried out.

The first is based on the vast knowledge gathered on the retention/recycling of mammalian Golgi enzymes within the Golgi apparatus; in particular the observation that the transmembrane domain together with the cytoplasmic domain and the stalk region in the lumen are mainly responsible for the retention (Nilsson and Warren 1994; Nilsson et al., 1996; Shima et al., 1997). We examined the possibility that a dGMII chimeric protein which lacked the catalytic domain (ΔdGMII) still localised in the Golgi apparatus of S2 cells as does the truncated form of NAGT1 (lacking its catalytic domain) (Shima et al., 1997). A construct, ΔdGMII-HA_3. consisting of the cytoplasmic domain, the transmembrane domain, the first 80 amino acids of the lumenal domain (corresponding to the stalk region) and a triple HA tag was transiently transfected into S2 cells. This chimeric protein was localised by immunofluorescence confocal microscopy after double labelling using anti-HA (to detect the product of the transfected construct) and anti-dGMII (to detect the native protein) as the primary antibodies. Near to perfect co-localisation of the two antibodies was observed (Fig. 5D) suggesting that the majority of ΔdGMII-HA_3 co-localised with endogenous dGMII. Moreover, a cryo-immunoEM experiment was carried out on these transfected cells using the anti-HA monoclonal antibody. This showed that the construct did reside in the Golgi apparatus of S2 cells, but also, as in the case of dGMII, in the ER

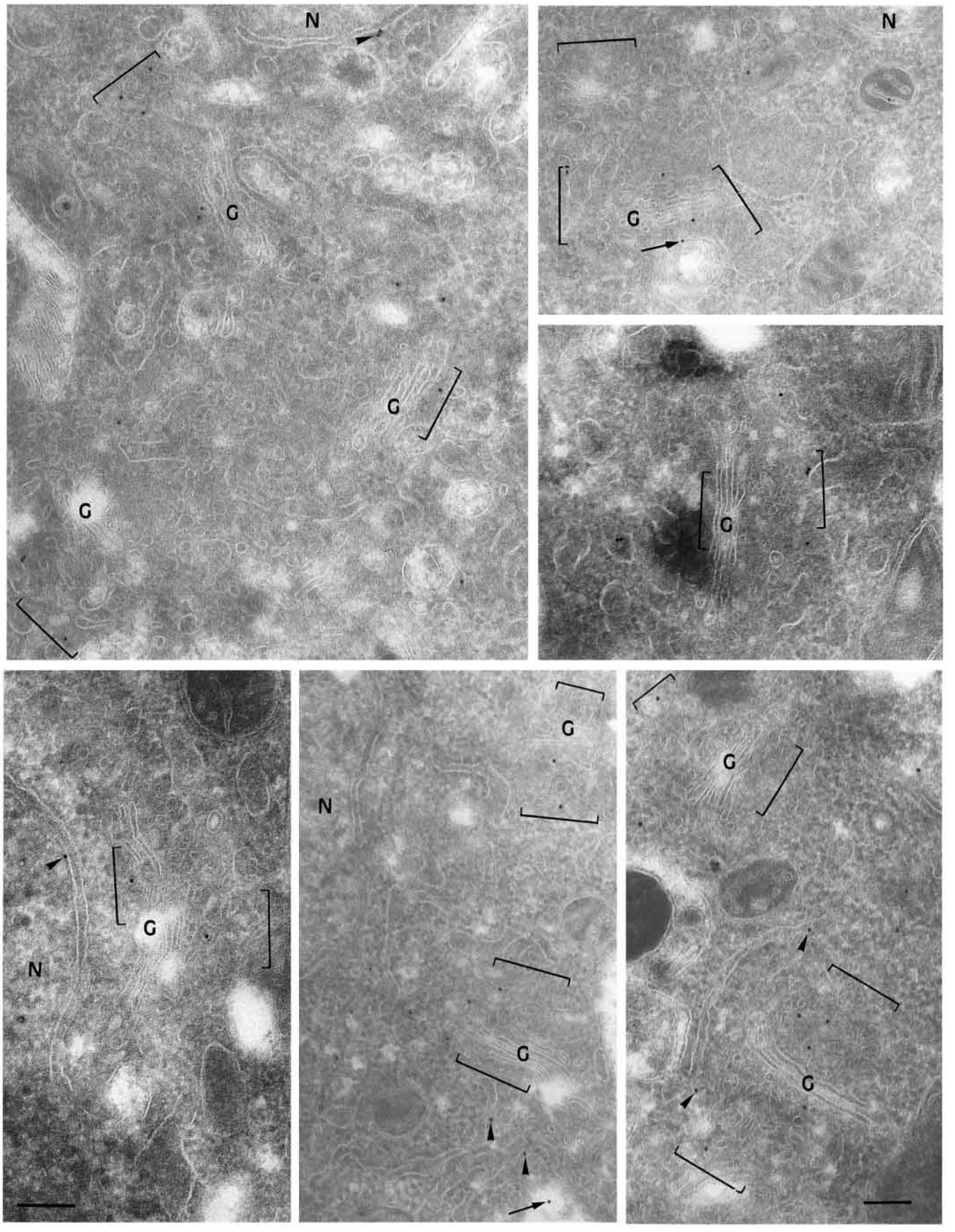

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Fig. 6. Cryo-immuno electron localisation of dGMII in S2 cells. Ultrathin frozen sections of *Drosophila* S2 cells were labelled with the anti-dGMII antiserum followed by Protein A coupled to 10 nm gold. As shown in the gallery of pictures, gold labelling was found in the Golgi apparatus, the endoplasmic reticulum and the endosomes. The area indicated between brackets represents the Golgi area. The Golgi stacks are indicated by G. The gold particles associated with the endoplasmic reticulum are indicated with filled arrowheads, and those associated with endosomes with thin arrows. N, nucleus. Bar, 200 nm.

cisternae (data not shown). This suggests that the ΔdGMII-HA_3 chimeric protein contained enough information to be localised to the Golgi apparatus of S2 cells to the same extent as the native protein.

In a second experiment we determined the localisation of a mammalian chimeric protein transiently transfected under similar conditions. We chose N-acetylglucosaminyl transferase I tagged with the myc epitope (NAGT1-myc, Nilsson et al., 1993). This had been shown to be a bona fide resident of the Golgi apparatus in HeLa cells (Nilsson et al., 1993). S2 cells transiently transfected with NAGT1-myc were fixed and a double immunofluorescence microscopy experiment was carried out using a monoclonal anti-myc antibody and anti-dGMII serum. Examination using a confocal microscope revealed that the two fluorophores generally co-localise (Fig. 5E), even though on some occasions the labelling for the chimeric protein was more restricted than for the native dGMII which showed diffuse staining throughout the cytoplasmic area. This suggests that the localisation signal of mammalian NAGT1 is able to operate in *Drosophila* S2 cells.

DISCUSSION

We have shown, by three lines of evidence, that the product of the *dGMII* gene is the *Drosophila* Golgi α-mannosidase II. First, the cDNA showed considerable sequence homology (41% identity and 61% similarity) to the mouse GMII. The greatest similarity is in the catalytic domain but the cytoplasmic and transmembrane domains share common features. Both are type II transmembrane proteins with a short cytoplasmic domain followed by a hydrophobic transmembrane domain. These are connected through a stalk region to the catalytic domain. The *dGMII* gene was cloned and sequenced and the genomic sequence described here shows that it has four introns with splice sites in keeping with the consensus splice sites found in *Drosophila*. We have demonstrated that the genomic sequence, with a shortened 3′UTR, transformed into flies has all the information necessary for it to be co-expressed with the native gene (Lockyer, 1995). However, a reporter sequence spliced onto the 5′ upstream sequence is not expressed normally, possibly because of the absence of introns (Lockyer, 1995). It remains to be seen how closely the *Drosophila* genomic sequence resembles the genomic sequence of the mammalian gene. Although it would be reasonable to propose that dGMII is the *Drosophila* homologue of mouse GMII, sequence similarity its not sufficient to demonstrate functional homology. It was necessary to demonstrate that the gene encoded a mannosidase and that this mannosidase was located in the Golgi apparatus.

Second, we have shown, here, that the *Drosophila* GMII exhibits mannosidase activity. The product of *Drosophila* cDNA expressed in CHOP cells hydrolyses PNP-mannose (the artificial substrate for α-mannosidase). It has a pH profile that is similar to other GMII's (pH optimum of 5.7 instead of 4.5 for the lysosomal mannosidases) and it is inhibited by swaisonine and by $CuSO_4$ as are other GMIIs.

Third, antibodies raised against a C-terminal peptide of dGMII were used to demonstrate that dGMII was a Golgi resident enzyme. They were first characterised using western blots of *Drosophila* embryo homogenates, membranes and cytosol. A single band was detected at a molecular mass corresponding to 124 kDa, when proteases were inhibited. This band was totally associated with the membrane fraction. The intensity of this band increased in transgenic p24.4.3 embryos that overexpressed dGMII. The same band was also detected in S2 cells (data not shown). When a truncated form of the protein was expressed using Baculovirus in *Spodopteran* cells, it was only detected in the cells that were infected. By all these criteria anti-dGMII was considered to be specific, and was used subsequently in the microscopy experiments.

We have shown that dGMII does reside in the Golgi apparatus of *Drosophila* S2 cells and *Drosophila* embryos using this anti-dGMII serum. The immunofluorescence pattern appeared as spots scattered around the nucleus reminiscent of the pattern in plant and yeast Golgi apparatus (Chappell and Warren, 1989; Henderson et al., 1994). In these organisms, the Golgi stacks appear as discrete and numerous vesicles which are not linked into a single ribbon-like organelle as in mammalian cells. Observation of S2 cell sections processed for conventional EM (not shown) and cryo-immuno EM (our results) revealed that 2-4 stacks of about 0.5 μm in diameter were present per cell section which were not linked into a longer structure. This Golgi organisation was consistent with the pattern observed with immunofluorescence using anti-dGMII. The immunolabelling of S2 cell cryosections using the anti-dGMII revealed that not only the Golgi apparatus but also the ER and the endosomes were labelled with gold particles. It could reflect the presence of newly synthesised dGMII in the

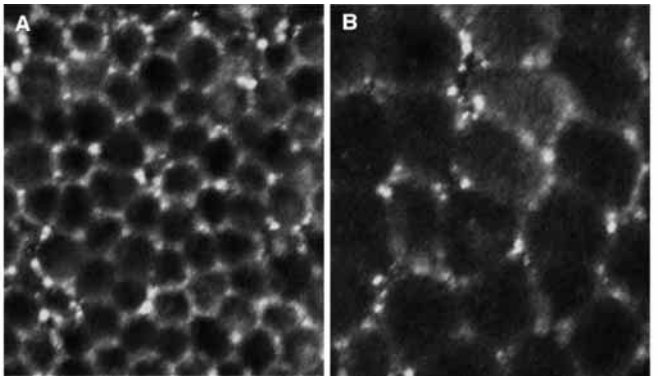

Fig. 7. Intracellular distribution of dGMII in *Drosophila* embryos. Six hour old *Drosophila* embryos were fixed, permeabilised, single labelled with antiserum to dGMII followed by an anti-rabbit antibody coupled to FITC and observed under a fluorescence confocal microscope (A low and B high magnification). The focus of the microscope was set to the bottom layer of cells. The pictures represent a single confocal section. Note that the pattern in A and B was punctate in a similar fashion to that in the *Drosophila* S2 cells.

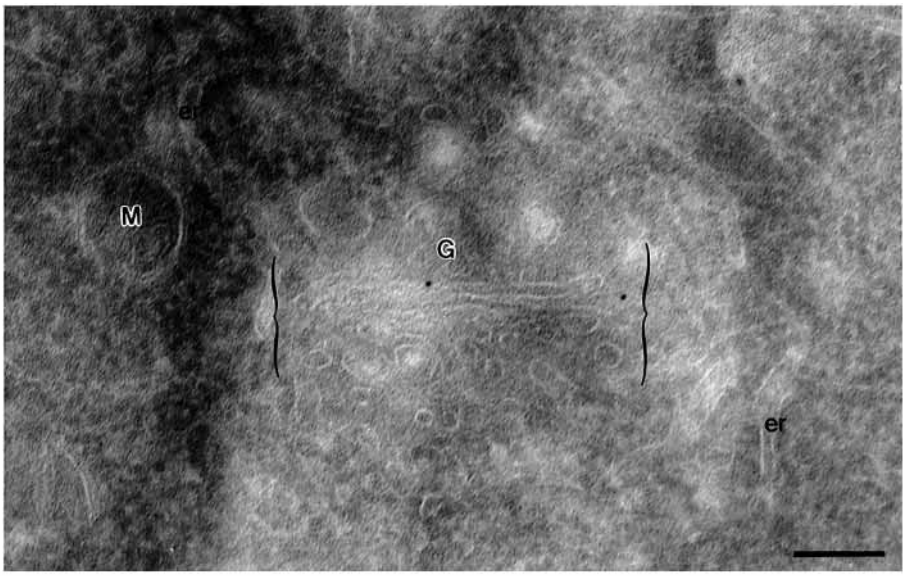

Fig. 8. Cryo-immuno electron localisation of dGMII in *Drosophila* embryos. Ultrathin frozen sections of 18 hour *Drosophila* embryos were labelled with the anti-dGMII antiserum followed by Protein A coupled to 10 nm gold. The cell shown belongs to the epidermis. Gold labelling was found in the Golgi apparatus. The area indicated between brackets represents the Golgi area. G, Golgi stacks; er, endoplasmic reticulum; M, mitochondria. Bar, 200 nm.

ER due to high rate of synthesis or slow exit from the ER. This has not been investigated in S2 cells and further work has to determine whether parameters such as transport, ER protein folding, glycosylation reaction could account for the presence of dGMII in the ER.

Alternatively, the retention of the enzyme in the *Drosophila* Golgi apparatus could be relatively weaker than in mammals where mGMII is tightly localised in the stack (Rabouille et al., 1995; Velasco et al., 1993).

In mammals, the localisation of Golgi resident enzymes involves a retention signal that is thought to reside in the transmembrane domain, the cytoplasmic and stalk region which acts to retain the Golgi resident enzymes once they reach their correct location (Nilsson and Warren, 1994; Gleeson, 1998; Munro, 1998; Shima et al., 1997). The fact that a ΔdGMII-HA_3 chimeric protein containing these three domains of dGMII showed a very similar distribution to native dGMII suggested that these three domains are sufficient for the localisation of dGMII in the Golgi apparatus.

These results could argue that dGMII is not as tightly localised in S2 as are the mammalian enzymes in mammalian cells. dGMII could recycle between the Golgi apparatus and ER. Golgi resident enzymes have been shown to slowly recycle to the ER (Hoe et al., 1995; Cole et al., 1998). The distribution of dGMII within the Golgi apparatus (where it is mostly found at the edge of the stack rather than tightly associated with the middle/trans-cisternae), and in the associated networks, (possibly the cis- and trans-Golgi networks) is consistent with this hypothesis.

dGMII did co-localise with other proteins that have been localised to the Golgi apparatus of mammalian cells (Gos28, syntaxin 5 and the delta subunit of the AP-3 complex). Using antibodies raised against the mammalian proteins, we were able to obtain, in *Drosophila* S2 cells, fluorescent patterns very reminiscent of the dGMII pattern (spots scattered around the nucleus). This suggests that cross reacting homologues of these proteins are expressed in S2 cells. Gos28 and syntaxin 5 are transmembrane proteins involved in crucial steps of fusion within the exocytic pathway and have been localised to the mammalian Golgi apparatus (Banfield et al., 1994; Nagahama et al., 1996; Hui et al., 1997). The anti-syntaxin 5 and anti-Gos28 antibodies used in this study were raised against the complete rat cDNAs expressed in bacteria. The *Drosophila* homologue of syntaxin 5 has been cloned and sequenced (Banfield et al., 1994) and shows 55% identity and 74% similarity with the rat sequence. *Drosophila* Gos28 has not yet been reported but the immunofluorescence data suggest that it exists and that the homology could be of the similar level. The antibody to the delta subunit of the AP-3 complex decorated the Golgi apparatus in mammalian cells (Simpson et al., 1997) as well as in *Drosophila* S2 cells (our results). There is a homologue to the delta subunit in *Drosophila* called garnet (Simpson et al., 1997; Ooi et al., 1997). *garnet* mutants show a defect in the eye pigmentation and the wild-type gene product has been implicated in the formation or the targeting of pigmentation granules.

Using these antibodies together with the anti-dGMII antiserum for a double labelling experiment of S2 cells, we found that co-localisation was extensive. This demonstrates that all these proteins share the same cellular distribution in the Golgi, ER and endosomes. We also transiently expressed the mammalian Golgi resident enzyme NAGT1 tagged with the myc epitope in S2 cells and found the fluorescent pattern was very similar to dGMII. We interpret this to mean that the the mammalian signal operating in NAGT1 for its localisation is as efficient in S2 cells as in mammalian cells.

Finally, *Drosophila* embryos were also labelled with the anti-dGMII antiserum. The fluorescence pattern of 6 hours old embryos (cellularisation finished) was similar to that observed in S2 cells. Spots were scattered around the nucleus as already reported using different markers (Ripoche et al., 1994; Stanley et al., 1997). Observation of older embryos by electron microscopy revealed that as in S2 cells, small stacks were scattered in the cytoplasm and that they were decorated by gold particles when labelled using the dGMII antiserum.

Taken together, these results support the idea that dGMII codes for the *Drosophila* functional homologue of the mammalian Golgi α-mannosidase II; that dGMII localised in the Golgi apparatus in *Drosophila* cultured cells and tissue and may be useful as a Golgi marker in *Drosophila*; and that the

Drosophila Golgi apparatus, although not organised as a single copy organelle, exhibits many similarities to mammalian cells in that it comprises stacked cisternae that contain glycosylation enzymes and SNAREs. Furthermore, in view of the widespread interest in the inhibition of the N-glycosylation in mammalian cells as a potential target for therapeutics, in particular for some cancers, the *Drosophila* homologue may well prove an appropriate model for the human enzyme, yet be more amenable to overexpression and structural studies by biophysical methods.

C.R., R.W. and MvdH were involved in the studies on gene product localisation; D.A.K., T.S. and D.R.R. were involved in gene product function and A.E.L. and D.B.R. in gene structure. The authors thank Dr Kelley Moremen (University of Georgia) for providing mouse mannosidase cDNA and pProtA+1; Dr Bozena Korczak (Glycodesign, Toronto) for transfecting the CHOP cells; Barbara Iafrate (OCI) for assisting with the mouse mannosidase construct; Dr Ian Jones (Oxford) for expressing dGMII in *Spodopteran* cells; Dr Stephanos Chritodoulou (Oxford) for work on the P4.24.3 transformants; Dr Tommy Nilsson (EMBL, Heidelberg) for the NAGT1-myc cDNA; Dr Thomas Söllner (Memorial Sloan Kettering Institute, New York) and Dr Margaret Robinson (Cambridge) for gifts of antibodies; Dr Terry Butters (Oxford) for his comments on the paper, and Dr Graham Warren (now at Yale University School of Medicine, New Haven) for use of his cryo-immunoEM equipment, gift of antibodies, and critically reading the manuscript. C.R. and MvdH acknowledge the receipt of a grant from the BBSRC(43/G09234). Work in the D.R.R. laboratory has been supported by the National Cancer Institute of Canada, as well as the Protein Engineering Network of Centres of Excellence.

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