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Cell-Free N-Glycosylation in *Dictyostelium discoideum:* Analysis of Wild-Type and Mutants Defective in Lipid-Linked Oligosaccharide Biosynthesis

Hudson H. Freeze, Petra Koza-Taylor, Jeffrey A. Jones, and William F. Loomis

Department of Medicine and Cancer Center, Cancer Biology Program (H.H.F., P.K.-T., J.A.J.) and Department of Biology (W.F.L.), University of California, San Diego, La Jolla, California 92093

N-glycosylation was measured in wild-type cell lysates of *Dictyostelium discoideum* and in two mutant strains that synthesize a truncated lipid-linked oligosaccharide, $Man_6GlcNAc_2$ lacking terminal mannose and glucose residues. Endogenous lipid-linked oligosaccharide (LLO) was transferred to octanoyl-Asn-[^{125}I]Tyr-ThrNH2 by membrane fractions. About 50% of the glycopeptide product remained associated with membranes. Taurocholate and saponin promoted and preserved glycosylation, but NP-40 and Triton X-100 did not.

Using this artificial assay, the rate and extent of transfer of the truncated lipid-linked oligosaccharide in extracts of the two mutant strains, HL241 and HL243, was reduced 5–10-fold relative to that of wild-type. The low activity found in the mutant strains appears to result from either reduced affinity of the truncated LLO for the transferase or from its improper topological localization in the membrane.

When protein N-glycosylation is measured in living cells it is nearly normal in HL241, but it is 3–4-fold decreased in HL243. Although the results of the in vitro and in vivo assays differ, they are not in conflict. Rather, they suggest that the static in vitro assay may be capable of revealing subtleties in the productive positioning of LLO and the oligosaccharyl transferase. The decrease in glycosylation seen in intact HL243 cells may be a consequence of the pleiotropic effects of the primary mutation rather than a direct result of the altered LLO structure. Genetic analysis showed that the mutation in HL241 is recessive, while the mutation in HL243 is dominant and prevents normal development. Thus, the two mutants share a lesion in lipid-linked oligosaccharide biosynthesis and in cell-free glycosylation, but differ in their in vivo glycosylation. Their primary defects are probably different.

Abbreviations used: LLO, lipid-linked oligosaccharide; GSBP, glycosylation site binding protein; CA1, common antigen 1; PBS, phosphate-buffered saline; Endo S, endoglycosidase S from *Dictyostelium;* mLE2, monoclonal antibody recognizing CA1.

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Key words: cell-free N-glycosylation, *Dictyostelium discoideum*, lipid-linked oligosaccharide biosynthesis, wild-type, mutant strains

N-glycosylation is a frequent post-translational modification of eukaryotic proteins. Synthesis occurs by the en bloc transfer of a preformed oligosaccharide from a lipid-linked donor ($Glc_3Man_9GlcNAc_2$-P-P-dolichol) to proteins with a properly presented tripeptide consensus sequence, Asn-X-Thr (Ser), where X can be any amino acid except Pro [1]. In vitro studies have used peptides containing this sequence to study the glycosylation process and the routing and secretion of glycopeptides in mammalian cells [2–7]. The oligosaccharyl transferase activity has been partially characterized in yeast [7] and hen oviduct [8]. A widely distributed glycosylation site binding protein (GSBP) has been identified by using a membrane permeant ^{125}I-labeled tripeptide coupled to a photoaffinity probe [3].

Various mutant strains of yeast and mammalian cells with specific defects in N-linked oligosaccharide biosynthesis have been described. These include defects in lipid-linked oligosaccharide (LLO) precursor biosynthesis, processing glycosidases, and glycosyl transferases (reviewed in [9–11]), but no mutants are known that lack oligosaccharyl transferase or GSBP. However, Tenner and Scheffler described a temperature sensitive mutation in CHO cells that was reduced in LLO transfer to protein [12]. We have recently described two mutant strains in *Dictyostelium discoideum*, HL241 and HL243, that fail to synthesize a carbohydrate antigenic determinant (CA1) found on N-linked oligosaccharides [13]. Although the primary defects are unknown, both strains are deficient in the mannosyl transferase(s) needed to synthesize a normal LLO and instead they produce a truncated LLO, $Man_6GlcNAc_2$ [14]. Here we describe cell-free glycosylation studies on the wild-type and these mutant strains using a membrane permeant ^{125}I-labeled tripeptide acceptor. In addition, we present biochemical and genetic evidence that the two strains have different primary defects and that one of these, a dominant mutation, prevents normal development.

MATERIALS AND METHODS

Materials

Radiolabels. [2-^{3}H]Mannose (20 Ci/mmole) was purchased from American Radiochemical Co. [6-^{3}H]Glucosamine (15 Ci/mmole), [^{35}S-labeled methionine/cysteine (TranS^{35}) (600 Ci/mmole), [^{14}C]leucine (300 mCi/mmole), and carrier-free Na^{125}I were purchased from ICN Radiochemicals (Irvine, CA). Octanoyl-Asn-Tyr-Thr NH2 tripeptide was a generous gift of Dr. Felix Wieland and Dr. James Rothman, Stanford University. The glycopeptide used as a substrate for Endoglycosidase S assays (^{14}C-Ac-Asn-$GlcNAc_2Man_6$) was prepared from ovalbumin glycopeptides acetylated with [^{14}C]acetic anhydride [15].

Strains. The origin and growth of strains AX4, HL241 and HL243 [13], and HL101 [16] have been described previously. In the original description of HL243, it was reported that the strain could complete development, however, only small aberrant fruiting bodies were observed while the great majority of cells failed to aggregate. Detailed quantitative morphogenic studies were not carried out at that time, but based on the genetic data presented here we believe that the primary mutation is the same as in the original isolate. Strains HL241.1 and HL243.1 were prepared during the course of the genetic analysis presented here.

Reagents. Concanavalin A-Sepharose was purchased from Pharmacia Fine Chemicals; Peptide:N-glycosidaseF was purified in this laboratory [17].

Antibody to GSBP. Rabbit antiserum IgG prepared against the glycosylation site binding protein (GSBP) and ribosome stripped hen oviduct membranes containing GSBP were generous gifts of Dr. William Lennarz, M.D. Anderson Hospital, Houston, Texas.

Methods

Preparation of Octanoyl-Asn-[^{125}I]Tyr-ThrNH2 tripeptide acceptor. Approximately 30 nmoles of the peptide was iodinated with 2 mCi of Na^{125}I and purified as previously described [2]. Approximately 25% of the label was incorporated and the specific activity of the purified product was approximately 2.5×10^7 cpm/nmole. The product was characterized by TLC as previously described [2].

Preparation of cell extracts. Log phase cells at $2–4 \times 10^6$ cells/ml were harvested by centrifugation washed once with breakage buffer (8.55% sucrose in 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM mercaptoethanol, 10 mM $MgCl_2$, 2 mM $MnCl_2$) and resuspended in the same buffer at 1.5×10^8 cells/ml. Cells were broken either by sonication using a Branson Model 450 sonicator at power setting 5 for a total of 10 s or by nitrogen cavitation at 1,000 psi. Both methods give total cell breakage. Total membranes were collected by sedimentation at 100,000g for 45 min, and subfractionated membrane vesicles, and ribosome stripped membranes were prepared as described [18] except that the membrane pellets were resuspended in breakage buffer prior to assay.

Oligosaccharyl transferase assay. Due to the lability of the oligosaccharyl transferase, cell lysates were assayed within 10 min of cell breakage, except where membrane fractions were collected. ^{125}I-Labeled peptide ($1–4 \times 10^5$ cpm) (usually 10–30 pmoles) was added to 0.2–1.5 mg of cell protein in 0.3 ml of breakage buffer and assayed for 10 min at 37°C except where different conditions are indicated. The samples were then heated at 100°C for 5 min, diluted to 1.5 ml with 0.01 M sodium phosphate buffer pH 7.5 and 0.15 M NaCl (PBS), and the precipitated protein removed by centrifugation at 10,000g on a Beckman microfuge. The supernatant, which contained all of the radioactivity, was loaded on a 2 cm column of ConA-Sepharose equilibrated in PBS and washed with 4×1.5 ml of PBS followed by 4×1.5 ml of PBS containing 0.1 M α-methylmannoside at 55°C. All samples were counted and the ConA-bound material was calculated as a percentage of the total counts recovered corrected for the level found in unincubated controls (0.1%). The columns themselves retained very little radioactivity following washing with the saccharide. All samples were counted on a Tracor gamma counter.

Characterization of the ^{125}I products of the transferase reaction. ^{125}I material bound to ConA-Sepharose was repurified by passing over Sep Pack C18, washing with water, and then eluting with 60% acetonitrile with a 60% recovery. A portion of this material was digested with 2 mU of EndoH for 16 h at 37°C in 0.01 M sodium phosphate buffer pH 6.5. Aliquots of the ^{125}I-labeled peptide, glycopeptide, or EndoH treated glycopeptide were analyzed by gel filtration chromatography on a 0.5×45 cm column of Sephadex G-25 equilibrated in 1 M acetic acid. All of the samples were somewhat retarded by interaction with the gel matrix, but this did not prevent resolution of the components (see Fig. 1). Alternatively, the samples were analyzed by TLC using Silica Gel-G plates developed in butantol/acetic acid/water (5:2:2)(v/v), until the

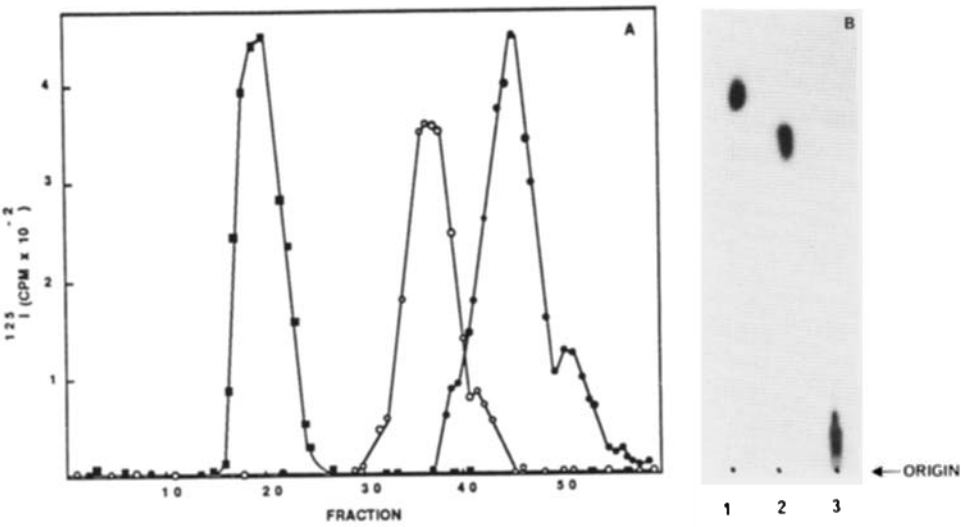

Fig. 1. Sephadex G-25 and TLC analysis of ^{125}I-labeled peptides and glycopeptide. **Panel A:** The samples prepared as described in the legend of Table I were analyzed on a 0.5 ×45 cm column of Sephadex G-25 equilibrated in 1 M acetic acid. The peptide substrate (●), or the repurified glycopeptide product was analyzed either before (■) or after (○) digestion with EndoH. **Panel B:** The same samples shown in panel A were also analyzed by TLC on Silica Gel G plates run in butanol/acetic acid/water (5:2:2:[v/v/v]) followed by autoradiography. Lane 1, control peptide; lane 2, glycopeptide treated with EndoH; lane 3, glycopeptide without EndoH treatment.

solvent reached the top of the plate. Following drying the plate was exposed to Kodak XAR film at −70°C with an intensifying screen.

GSBP Assay. The presence of GSBP was probed using Western blot analysis. Approximately 50 μg of membrane fractions separated on discontinuous sucrose gradients [18] that contained both oligosaccharyl transferase and α-glucosidase II activity were run on 7.5% polyacrylamide gels, blotted onto nitrocellulose, and developed using HPR secondary antibody to the rabbit IgG [23]. Ribosome stripped microsomes from hen oviduct (50 μg) served as the positive control. Preliminary dot blot analysis used up to 25 μg of cell homogenate, 100,000g supernatant or total cell membranes.

Labeling of cells. Log phase cells of strains AX4, HL241, and HL243 were suspended at 1×10^7 in 1 ml of HL-5 growth medium and each was incubated with the following combination of radioisotopes: A, 5 μCi/ml [^{3}H]Mannose and 1 μCi/ml of either [^{35}S]methionine/cysteine or [^{14}C]leucine; B, 5 μCi/ml [^{3}H]GlcN and 1 μCi/ml of either [^{35}S]methionine/cysteine or [^{14}C]leucine. Labeling was continued for 30 min with shaking at 22°C. The cells were sedimented by centrifugation, washed three times with 5 ml of 0.01 M sodium phosphate buffer, pH 6.5, and lysed in 0.5 ml of 0.01 M sodium phosphate buffer, pH 6.5, and lysed in 0.5 ml of the same buffer containing 0.05% Triton X-100. A portion of the sample was brought to 10% trichloroacetic acid and heated for 5 min to destroy LLO. The precipitates were then washed and counted. In some cases the TCA precipitated material was solubilized in a small volume of 1% SDS with heating, diluted in PBS, and then chromographed on ConA-Sepharose. The columns were 2 × 0.5 cm and the sample was loaded in 1.5 ml of PBS, pH 6.5, and washed with 4 × 1.5 ml

of PBS followed by 4 × 1.5 ml washes of the same buffer containing 200 mM α-methylmannoside at 55°C.

A portion of the TCA precipitate was solubilized in 1% SDS, diluted and digested with 100 μg/ml of Pronase at 55°C. The Pronase was inactivated by heating at 100°C and the digest was analyzed on ConA-Sepharose. Another portion of the solubilized TCA precipitate was diluted and digested with Peptide: N-glycosidaseF to liberate N-linked oligosaccharides that were separated from the nonreleased material by chromatography on Sephadex G-50 [17,19].

***In vivo* synthesis of LLO.** Actively growing cells were incubated with 0.3 mCi of [^{3}H]mannose in 10 ml of HL-5 medium at 1 × 10^7 cells/ml. Aliquots were taken at various times and the cells collected by centrifugation, washed twice with 0.01 M NaPO4 buffer, pH 6.5, and the LLO extracted in 10:10:3 chloroform/methanol/water as described previously [14]. The oligosaccharide was freed from the lipid by mild acid hydrolysis [20] bound to ConA-Sepharose, eluted with 100 mM α-methylmannoside and counted.

Degradation of the LLO. Cells were labeled as above with 0.3 mCi of [^{3}H]mannose for 1 h in HL-5. The radioactive medium was replaced with HL-5 containing 1 mg/ml of mannose, and the incubation was continued for up to 1 h. At various times the cells and the medium were removed and the LLO was isolated and quantified as above.

Endoglycosidase S assay. Endo S is a soluble enzyme in *Dictyostelium* and cleaves high mannose-type oligosaccharides from glycopeptides [15]. Extracts were prepared from each cell line by lysing the cells in 0.05 M $NaPO_4$ buffer containing 0.1% Triton X-100. Fifty to two hundred micrograms of protein in 200 μl of the above buffer were incubated with 10,000 cpm of [^{14}C]Ac-Asn-GlcNAc$_2$-Man$_6$ glycopeptide derived from ovalbumin [15]. At the end of the reaction, the mixture was boiled to inactivate the enzyme, and the mixture was diluted to 1.5 ml in PBS and analyzed by ConA-Sepharose chromatography. Each sample was washed with 3 × 1.5 ml of PBS to elute the cleaved [^{14}C]Ac-Asn-GlcNAc and then with 100 mM α-methylmannoside to elute the intact glycopeptide. Activity was calculated as the percentage of radioactivity not bound to the lectin.

α-Glucosidase II assay. The endoplasmic reticulum associated α-glucosidase II, which is involved in oligosaccharide processing, was assayed as previously described [20].

Genetic analysis of mutant strains. Derivatives of strains HL241 and HL243, which carry recessive mutations that prevent growth at 27°C, were isolated [22] and named HL241.1 and HL243.1, respectively. The temperature sensitive strains still retained their original phenotypes of 1CA1 expression and developmental competence. Diploids were isolated from the two strains or between one of them and a growth temperature sensitive strain that permits growth in the presence of 2% methanol. Only the diploids are capable of growing at the restrictive temperature [22]. Haploid segregants of DL272 were selected either for their ability to form mature fruiting bodies or for growth in the presence of 2% methanol. The presence of antigenic determinant CA1 was assessed by slot blot analysis on nitrocellulose [23] and by the ability of antibody mLE2 (CA1) to precipitate N-acetylhexosaminidase activity [24]. Both assays gave comparable results.

RESULTS

Assay and Product Characterization

A variety of modified peptides are available to study N-glycosylation in vitro [2–7]. We chose octanoyl-Asn-[^{125}I]-Tyr-ThrNH_2 as an acceptor because of its high specific activity, membrane permeability, use in glycosylation and routing studies in intact cells, and potential for inhibiting N-glycosylation in intact cells [2]. When crude cell lysates are incubated with ^{125}I-labeled tripeptide up to 8.5% of the material can be glycosylated as shown by its binding to ConA-Sepharose. Purification of the bound material on a C18 Sep-Pack column followed by reapplication to ConA-Sepharose shows that 98% of the material binds and is eluted by α-methylmannoside. Endoglycosidase H (Endo H) digestion of the product abolishes 95% of this binding (data not shown). Analysis of the ^{125}I-labeled peptide, glycopeptide, or EndoH digested glycopeptide on Sephadex G-25 also shows that ^{125}I-labeled material that binds to ConA-Sepharose is larger than the ^{125}I-labeled peptide, and that Endo H digestion reduces the size to nearly that of the original peptide (Fig. 1, panel A). TLC analysis (Fig. 1, panel B) of the same samples indicates a similar finding [2]. These results show that the ^{125}I labeled peptide is radiochemically homogeneous and that it is an acceptor of N-linked oligosaccharides in *Dictyostelium*, as previously shown by others using different systems [2].

N-glycosylation occurs exclusively on the luminal side of the ER [25] where oligosaccharyl transferase transfers the oligosaccharide from endogenous LLO to acceptor peptides or endogenous proteins [1]. To determine the localization of the glycosylation activity in *Dictyostelium*, cells were broken by sonication or by nitrogen cavitation and assayed for activity in crude lysates, total membrane fraction, 100,000g supernates, or in reconstituted membrane-supernatant mixtures. The results shown in Table I indicate that both cell breakage methods give essentially the same results with nitrogen cavitation yielding slightly higher activity. All of the activity resides in the membrane fraction, and no stimulation occurs upon reconstituting equal amounts of the 100,000g supernate and membrane fractions. This suggests that no essential component is solubilized during the breakage by either method.

Significant glycosylation occurs at 5°C as well as at 20°C and 37°C. At all temperatures, 8–11% of the hydrophobic peptide and about 50% of the glycopeptide is

TABLE I. Distribution of Transferase Activity[a]

	Glycosylation activity[b]			
		(100,000g fraction)[c]		
Breakage method	Unfractionated lysate	Pellet	Super	1:1 Mix pellet and super
Sonication	0.435	0.428	0.0	0.185
Nitrogen cavitation	0.540	0.481	0.0	0.270

[a]Log phase AX4 cells were harvested by centrifugation, washed in breakage buffer, and resuspended at 1.5 × 10^8 cells/ml in the same buffer. They were sonicated or broken by nitrogen cavitation as described in Methods.

[b]Activity is expressed as pmoles of peptide converted to glycopeptide in 10 min at 37°C.

[c]The lysates were used directly or were fractionated by centrifugation at 100,000g for 45 min. The resulting membrane pellet was resuspended in the same volume of breakage buffer and assayed. The supernate was either used directly for assay or mixed 1:1 with an equal volume of resuspended membranes prior to assay. All assay volumes were the same. Thus, without activators or inhibitors present, the activity expected in the mixture would be the average of the separate activities.

membrane associated. The same results were seen for homogenates prepared by nitrogen cavitation using wild-type or HL241 cells (data not shown). These results suggest, but do not prove, that about half of the glycosylation occurs within the membrane vesicles and that it is not simply hydrophobic association of the peptide with membranes. Similar results have been observed by others using intact sealed vesicles and membrane permeant labeled peptides to measure glycosylation [25]. When incubations are carried out in the presence of sodium taurocholate, 75% of the peptide and essentially all of the labeled glycopeptide is solubilized.

Low concentrations (0.02%) of saponin and sodium taurocholate stimulated transferase activity while higher concentrations (0.16%) were inhibitory. Low concentration (0.02%) of deoxycholate inhibited 50% of the activity, but NP-40 and Triton X-100 abolished activity even at low concentrations (Table II).

Comparison of Wild-Type and Mutant Strains

The in vitro glycosylation activity of mutant strains HL241 and HL243 is only about 10–15% that of wild-type, as shown in Figure 2. The results shown in Figure 3 indicate that the initial rate and extent of glycosylation is considerably lower in the mutants than in the wild-type at either 5°C or 37°C. Preincubation of the extracts for 4 min at 5°C prior to the addition of acceptor peptide and assay at this temperature showed little loss of activity in either the wild-type or the mutants (data not shown). Longer incubations of each of the extracts on ice showed that all were labile and lost activity with a half-life of 20–30 min (Fig. 4). Therefore, all assays were conducted within 10 min of cell breakage. Since the glycosylation system is equally stable in each strain, these data suggest that a component(s) becomes rate limiting.

Availability and Km of Peptide Acceptor

If the acceptor peptide is rapidly degraded during the incubation, a second addition of peptide later in the assay should noticeably increase glycosylation activity, but this did not occur in the wild-type or either of the mutants, suggesting that the amount of peptide does not become limiting (data not shown). Alternatively, if the peptide were less membrane permeant in the mutants, this would also yield lower activity. This also does not appear to be the case, since the proportion of both peptide and glycopeptide associated with the vesicles at 5°C or 37°C in HL241 is the same as the wild-type (data not shown).

TABLE II. Effects of Various Detergents on Glycosylation Activity*

Detergent	Percent (v/v)	Percent of control activity
Control	—	100
Saponin	0.02	200
	0.16	25
Tauracholate	0.02	190
	0.16	25
Deoxycholate	0.02	52
NP-40	0.015	12
	0.10	0
Triton X-100	0.10	0

*Various detergents were added at the concentrations indicated (v/v) to 700 μg of cell protein and assays carried out as described in Methods. Control value was 0.26 pmoles of ^{125}I-peptide glycosylated.

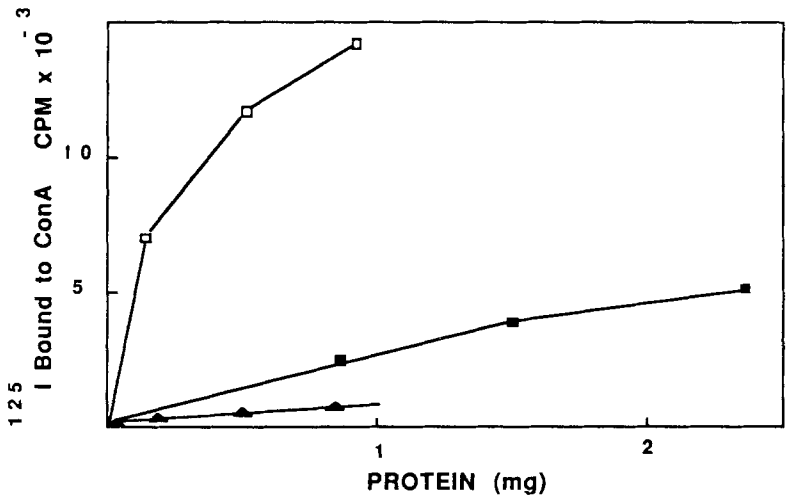

Fig. 2. Glycosylation activity of wild-type and mutant strains. Cell lysates were prepared for each strain and various amounts of protein were incubated with ^{125}I-labeled peptide. Assays were performed as described in Methods. AX4, (□); HL241 (■), HL243 (▲).

Yet another possible explanation for the lower activity in the mutant strains is that the oligosaccharyl transferase or GSBP has an altered Km for the acceptor peptide. As shown in Figure 5, this is not the case; the Km of the peptide is about 2.5 micromolar in each strain. Thus, the concentration of the acceptor is not saturating in this assay. The Vmax is calculated to be 17.8, 3.8, and 2.1 pmoles/mg protein for AX4, HL241, and HL243, respectively.

Search for GSBP

A soluble, intraluminal glycosylation site binding protein (GSBP) that recognizes the Asn-X-Thr consensus sequence [3] is found in *Drosophila* and a variety of mammalian cells, but not in yeast [1]. Lower glycosylation activity in our mutant strains could result from a deficiency in the putative GSBP.

To determine this, ribosome-stripped ER fractions of wild-type cells were probed with a polyclonal antibody against the GSBP. No cross-reacting protein was detected in Western blots of SDS-polyacrylamide gels, using stripped hen oviduct microsomes as a positive control. Similarly, dot blot analysis of several other membrane and soluble fractions also gave negative results (data not shown). These data suggest that *Dictyostelium* does not contain a GRBP that is similar enough to be recognized by the polyclonal antibody against hen GSBP [3].

Temperature Shift

Another reason for the lower activity in the mutant strains could be that the amount of LLO "available" for transfer is limited. Evidence consistent with this idea is presented in Figure 6, although other interpretations are also possible. The results show that the rate of glycosylation decreases with time in both strains, even at 5°C, but when the temperature is shifted to 20°C, the rate increases once again. It appears that the rate limiting component is depleted after 5 min at 5°C, but is replenished when the

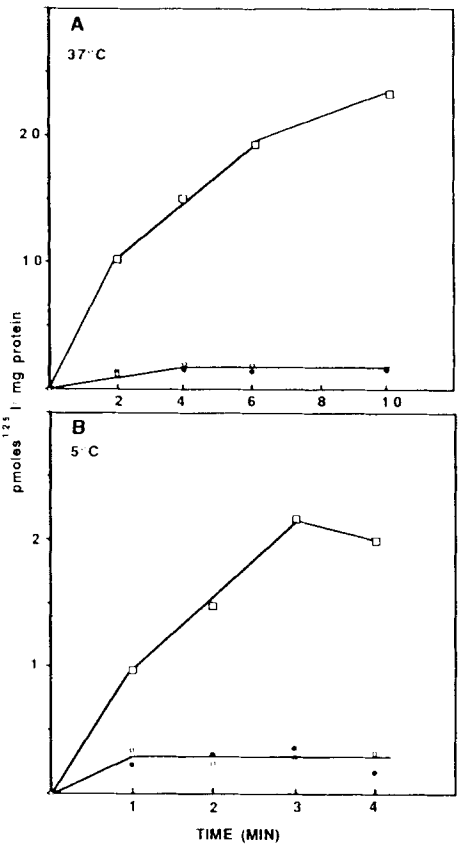

Fig. 3. Glycosylation activity in wild-type and mutant strains at different temperatures. Extracts were prepared from each strain (AX4, □; HL241, ○; HL243, ●) and incubated with ^{125}I-labeled peptide at either 37°C (**panel A**) or 5°C (**panel B**). At various times, the reactions were terminated and the products analyzed.

temperature is raised to 20°C. These results are consistent with the LLO being a rate limiting component, since limited diffusion of the LLO at 5°C would lead to its rapid depletion, and warming would increase the mobility and replenish the supply of LLO available to the transferase. This pattern is seen for both strains, but the rate of glycosylation at 20°C is still much greater in the wild-type than in the HL241. Since the rate and extent of glycosylation is much less in strain HL241 than AX4, it appears that the LLO donor is more severely limited in the mutant strain. The lower rate of glycosylation could also be explained by a difference in the Km of $Man_6GlcNAc_2$ LLO in the mutants compared to the wild-type, $Glc_3Man_9GlcNAc_2$ LLO. All attempts to supplement and stimulate the in vitro system by the addition of exogenous LLO were unsuccessful, so we could not determine Km differences or if LLO became limiting during the assay. Instead, we approached the question by measuring the relative amount and rates of synthesis and degradation of LLO in intact cells.

In vivo Synthesis and Turnover of LLO

We labeled intact cells with [^{3}H]mannose for various times and isolated the LLO. As shown in Figure 7, panel A, the relative amount of ^{3}H incorporated and its rate of

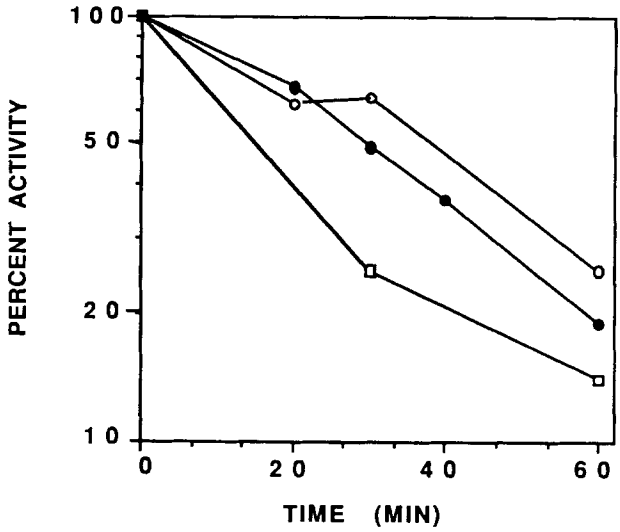

Fig. 4. Stability of glycosylation activity. Extracts were prepared from wild-type (□) and mutant strains (HL241, ○, or HL243, ●) were assayed immediately (0 time) or incubated at 0°C for various periods of time. At each point an aliquot was removed and assayed for glycosylation activity at 37°C for 10 min. Results are expressed as a percent of zero time activity.

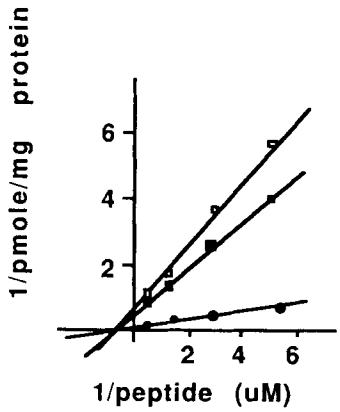

Fig. 5. Substrate dependence of transferase. Cell lysates were prepared and incubated with the same amount of ^{125}I-labeled peptide and increasing amounts of unlabeled peptide. The activity of each strain was determined as described in Methods. AX4, ○; HL241, ■; HL243, □.

incorporation into LLO was nearly the same in each strain. We did not determine the specific activity of the LLO in each strain, and therefore, could not determine the actual amount synthesized. However, we previously showed that the amounts of Dol-P and Dol-P-Man are the same in the mutants and in the wild-type [14]. We assume that the specific activities of the precursor pools are the same in each strain, and that the radioactivity incorporated into LLO is a reliable measurement of the amounts synthesized. The rate of turnover of LLO was measured by pulsing the cells for 1 h with [^{3}H]mannose followed by a variable period of chase. The rate of turnover of LLO

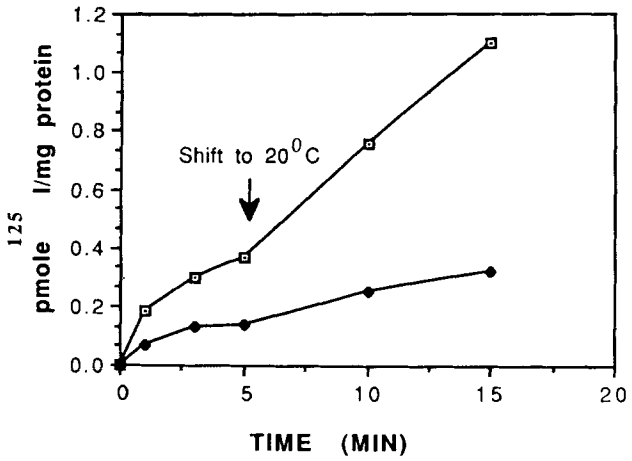

Fig. 6. Effects of temperature shift on glycosylation activity. Cells of strains AX4 (⊡) or HL241 (♦) were prepared in breakage buffer and approximately 1 mg of cell protein was incubated with 15 pmoles of ^{125}I-labeled peptide at 5°C. After 10 min the remaining tubes were shifted to 20°C and the incubation continued.

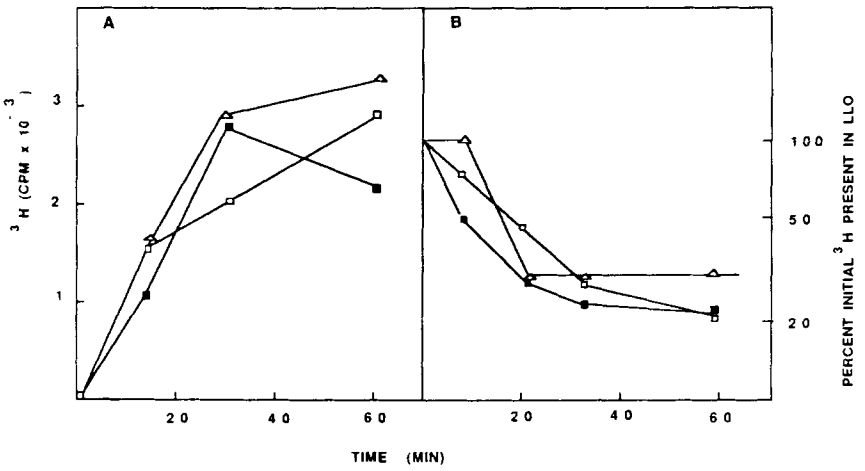

Fig. 7. Rate of synthesis and turnover of LLO in the wild-type and mutant strains. **Panel A:** Cells of each strain were continuously labeled with [^{3}H]mannose and at various times samples were removed and analyzed for radiolabeled LLO as described in Methods. **Panel B:** Cells were labeled for 1 h with [^{3}H]mannose and transferred to unlabeled medium. LLO was extracted at various times and the rate of turnover determined relative to the beginning of the chase. AX4, □; HL241, ■; HL243, △.

(degradation plus protein glycosylation) was not significantly different in cells of the different strains (Fig. 7, panel B). Thus, the relative amount of LLO, its rate of synthesis and turnover, cannot account for the differences seen in cell-free glycosylation.

N-Glycosylation In Vivo

The results presented to this point suggest that the lesion in LLO biosynthesis, shared by the mutant strains, is responsible for the low level of in vitro glycosylation. This

is probably due to a limitation in the amount of LLO available to the transferase, or to an altered Km. However, the in vitro assay probes a relatively static system in which there may be limited flow of critical, nonrenewable components. When measuring glycosylation in vivo, there is a constant flux of small metabolites, macromolecules, and metabolic energy that could yield different results when comparing the different strains. ^{125}I-labeled tripeptide acceptor was not glycosylated in intact cells, so each strain was double-labeled with [^{3}H]glucosamine and [^{35}S]Met (or [^{14}C]Leu) for 30 min. The proteins were precipitated with TCA or re-solubilized and chromatographed on ConA-Sepharose. Cells of strain HL241 incorporated about 90% as much of the [^{3}H]GlcN as wild-type cells when normalized to protein synthesized, but cells of strain HL243 incorporated 3.5-fold less (Table III). The majority (65–72%) of the ^{3}H-labeled material was released by digestion with PNGaseF, showing that it was present in N-linked oligosaccharides. A similar glycosylation pattern was also seen when ConA-Sepharose bindable glycopeptides were prepared from each of the strains. If the labeling is performed with [^{3}H]mannose instead of [^{3}H]glucosamine, similar results are also seen. This suggests that protein glycosylation is nearly normal in HL241, but it is considerably reduced in HL243. Furthermore, it suggests that although the two mutant strains share a similar lesion in LLO biosynthesis and in vitro glycosylation, they may not be identical.

Genetic Analysis

To determine whether the mutations in the two strains were in the same locus, we conducted a genetic analysis. Strain HL241 produces mature fruiting bodies while strain HL243 cannot initiate aggregation, the first step in fruiting body construction. Derivatives of strains HL241 (HL241.1) and HL243 (HL243.1) that are temperature sensitive for growth at 27°C were crossed with strain HL101 that is wild-type for the expression of the carbohydrate antigenic determinant CA1, makes fruiting bodies, and carries an independent mutation resulting in growth temperature sensitivity. This strain also carries a recessive marker, acrA, that allows growth in the presence of 2% methanol. Strain DL271, a diploid formed from strains HL241 and HL101, develops normally and

TABLE III. Analysis of Glycoproteins and Glycopeptides Metabolically Labeled With ^{3}H Sugars*

	Incorporation of ^{3}H		
Sample	AX4	HL241	HL243
Total TCA precipitates			
GlcN	100	91	31
Man	100	81	38
ConA-Sepharose bound			
GlcN	100	88	26
Man	100	100	26
Pronase glycopeptides			
GlcN	100	81	26
Man	100	66	22
Percent released by PNGaseF			
GlcN	72	68	74
Man	80	81	74

*Labeling and analysis of samples was performed as described in Methods. Wild-type (control) values were set to 100 and all values were normalized to [^{35}S]Met incorporation. The percentage of ^{3}H released by PNGaseF digestion was determined by chromatography on Sephadex G-50 as previously described [19].

TABLE IV. Genetic Analysis of HL241 and HL243

Strain	CA1[a]	Development[b]
Haploid		
AX4	+	+
HL101	+	+
HL241	–	+
HL243	–	–
Diploid		
DL271 (HL241.1/HL101)	+	+
DL272 (HL243.1/HL101)	–	–
DL273 (HL241.1/HL243.1)	–	–
Haploid segregants of DL272		
S1	+	+
S2	+	+
S3	+	+
S4	+	+
S5	–	–
S6	–	–
S7	–	–
S8	–	–
Haploid segregant of DL273		
S1	–	–

[a]Presence of CA1 determined as described in Methods.
[b]Development was assessed by the ability to form fruiting bodies (+) failure to aggregate (–).

synthesizes CA1 (Table IV). Therefore, the mutation that affects the synthesis of CA1 must be recessive. On the other hand, strain DL272, a diploid formed from strains HL243 and HL101, is blocked in aggregation and does not carry CA1. When strain DL272 segregates haploid recombinants that are able to develop normally (S1–S4), all contain CA1. Conversely, when DL272 was grown in the presence of 2% methanol, none of the segregants (S5–S8) developed or expressed CA1. Likewise, a diploid formed from stains HL241 and HL243, DL273, neither aggregates nor forms CA1. These results show that strain HL243 carries a dominant mutation that blocks aggregation as well as a dominant block in the synthesis of CA1. Since dominant mutations are much less prevalent than recessive mutations in this and other haploid organisms, it is very likely that the aberrations in morphogenesis, LLO, biosynthesis, and glycosylation are pleiotropic consequences of the same dominant mutation. The co-segregation of these phenotypes in recombinant haploids derived from strain DL272 further supports this conclusion. These results indicate that the mutations in HL241 and HL243 are different from each other. Moreover, the lesion in HL243 is not on linkage group IV since CA1 expression and β-hexosaminidase activity do not co-segregate in haploid recombinants.

DISCUSSION

We have shown that *Dictyostelium* cell lysates can transfer oligosaccharides from endogenous LLO to octanoyl-Asn-[^{125}I]Tyr-ThrNH_2 and that the glycopeptide product binds to ConA-Sepharose and is totally sensitive to EndoH digestion. All of the activity is associated with membranes and the reaction is neither activated or inhibited by factors solubilized during cell breakage. Experiments designed to identify a glycosylation site binding protein (GSBP) using antibodies to hen GSBP failed to recognize a homologous

protein in *Dictyostelium* either because these cells do not contain this protein or because the GSBP in *Dictyostelium* is sufficiently different that is does not cross-react with antibodies to hen GSBP (Data not shown).

Mutant strains HL241 and HL243 do not produce an antigenic determinant CA1, which is found on sulfated N-linked oligosaccharides [13]. The loss of the determinant results from both strains synthesizing a truncated LLO, $Man_6GlcNAc_2$ due to the absence of a mannosyltransferase [14]. In the present study we found that the mutants are much less active in the cell-free glycosylation assay. It is unlikely that the oligosaccharyl transferase protein itself is directly altered in both mutants since the loss of mannosyl transferase activity involved in LLO biosynthesis should not affect oligosaccharyl transferase. We have shown that it is not due to differential stability of the system, depletion, or unavailability of the acceptor, or to a change in its Km. It is more likely that the lowered activity is a direct consequence of the altered structure of the LLO. If the lower in vitro glycosylation activity in the mutants is due to their production of $Man_6GlcNAc_2$, it could result from a lower amount of LLO available for transfer or from a lower affinity for the truncated LLO. We were unable to directly test whether the amount of LLO was rate limiting in the cell-free assay, but the total amount of LLO, as well as its rate of synthesis and degradation in intact cells, are nearly the same in all three strains.

The results of these measurements cannot address the important question of the amount of LLO available in the critical microenvironment of the oligosaccharyl transferase itself. The in vitro assay generates a freeze-frame picture of a relative static system in which limited mobility of important components and the absence of metabolic energy could be highly significant. It may be that the position of the $Man_6GlcNAc_2$ LLO in relation to the transferase is different in the mutants compared to the wild-type. Based on previous results on the in vitro synthesis of LLO in HL241 and HL243 [14] and by analogy with the biosynthesis of LLO in mammalian cells, the LLO in these strains should normally face the luminal, not the cytoplasmic side of the ER, and be accessible to the oligosaccharyl transferase. On the other hand, perhaps the $Man_6GlcNAc_2$ LLO in the mutants is not delivered to the transferase in an appropriate and useful fashion. Clearly, a component becomes limiting when the reaction is carried out at 5°C and it can be replenished by increasing the temperature to 20°C. A possible explanation for this finding is that a 5°C, LLO becomes rapidly depleted and it is not replenished because of limited diffusion at this temperature. When the temperature is increased to 20°C, the LLO has greater mobility in membranes and can replenish the glycosylation machinery. Studies in yeast strains impaired in various steps of glycosylation show that essentially all of the intermediate biosynthetic forms of LLO can be transferred to protein, including those thought to be synthesized on and oriented toward the cytoplasmic face of the ER [1,30,33]. LLO bearing different oligosaccharide chains may shuttle between the luminal and cytoplasmic faces of the RER at different rates. It is clear that our knowledge of the influence of subtle differences in substrate structure and the nature of the intimate topological relationships of the LLO and the oligosaccharyl transferase is insufficient. It is difficult to make confident predictions about the results of in vitro glycosylation activity using various endogenous LLO substrates.

Another explanation for lower activity is that the Km for the $Man_6GlcNAc_2$ LLO could be higher than the wild-type $Glc_3Man_9GlcNAc_2$ LLO. Previous studies suggest that the structure of the LLO species influences its transfer to endogenous proteins or

added peptides [7,26,27]. In vitro studies using soluble oligosaccharyl transferase partially purified from mammalian cells show little difference between the Km for Dol-P-P-$GlcNAc_2$ and Dol-P-P-$GlcNAc_2Man_9Glc_3$ [7], while the Dol-P-P-$GlcNAc_2Man_9$ is not transferred to the peptide acceptors. Other studies in intact cells show preferential transfer of glycosylated oligosaccharides to proteins [26,29]. An alteration in Km or less "available" LLO are both plausible explanations for the lower glycosylation activity observed in the mutant strains.

Glycosylation in intact cells is reduce 3–4-fold in only HL243, not in HL241, compared to wild-type. We interpret this to mean that in vivo, $Man_6GlcNAc_2$ can be used nearly as efficiently as the LLO in wild-type cells, i.e., it is not rate limiting because the system is dynamic and self-replenishing. This is not true with the in vitro assay. This result suggested that HL243 has additional lesions in normal glycosylation that might be unrelated simply to the structure of the LLO. Since the rate of turnover of the LLO is about the same in all of the strains, it suggests the LLO from HL243 may be degraded rather than transferred to protein. *Dictyostelium* contains an EndoH-like activity, EndoS, that appears to be located in the cytoplasm [15]. It is possible that hyperactivity of this enzyme could lead to enhanced degradation of the LLO [31,32]. However, direct assay of this activity showed it was the same in all three strains, and therefore, could not account for the enhanced degradation. We also considered the possibility that HL243 carried a mutation that affected other resident ER proteins besides the oligosaccharyl transferase and the LLO biosynthetic enzymes. To examine this we assayed the activity of oligosaccharide processing α-glucosidase II, the only other known ER marker in *Dictyostelium,* but found its activity was the same in all strains.

The differences between the two mutant strains was confirmed by a genetic analysis. It is very likely that the primary defects in the two mutant strains are different since the mutation in strain HL241 is recessive while that in strain HL243 is dominant with respect to both glycosylation and morphogenesis. The rarity of dominant mutations combined with the co-segregation of the phenotypic characteristics suggests that the lesion in strain HL243 has pleiotrophic effects on the synthesis of an altered LLO, reduced in vivo and in vitro glycosylation, and morphogenesis. The mutation in strain HL241 results in an alteration in LLO structure and reduced in vitro glycosylation, but has no effect on in vivo glycosylation or morphogenesis.

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