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Biochimica et Biophysica Acta 1281 (1996) 15–22

BBA Biochimica et Biophysica Acta

Subcellular distribution of 'intersecting' β-*N*-acetylglucosaminyltransferase in *Dictyostelium discoideum*. A likely marker for the Golgi apparatus [1]

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Received 24 July 1995; revised 27 November 1995; accepted 5 December 1995

Abstract

Glycoprotein processing in *Dictyostelium discoideum* is characterized by enzyme catalyzed steps not reported in other organisms. One of these is the formation of a β 1 → 4 linkage between GlcNAc and the mannose linked to the core mannose in the α 1 → 6 position of *N*-glycosides. A simple and sensitive assay for this GlcNAc transferase activity, using a tri-mannose acceptor and a low concentration of UDP-GlcNAc, was developed. Homogenates of the organism were subjected to sub-cellular fractionation by centrifugation in discontinuous sucrose gradients. The specific activity was enriched 4–5-fold in a crude membrane fraction. The transferase was purified 10–12-fold in a membrane fraction that bands on top of 1.1 M sucrose. This fraction was also enriched in nucleotidyldiphosphatase. The enriched fraction was deficient in glucose-6-phosphatase, an endoplasmic reticulum marker. Approx. 80% of the transferase activity was latent, and unavailable to protease. Purified membranes were either subjected to phase separation in Triton X-114, or sodium carbonate extraction or sonication. In each case, the transferase behaved as an intrinsic membrane protein. Several secreted and lysosomal proteins are modified by the enzyme. These data support the idea that the GlcNAc transferase is present as an integral Golgi membrane protein and that at least the catalytic center of the transferase is on the lumenal side of the vesicles

Keywords: Golgi; Marker enzyme; GlcNAc transferase; Nucleotidyldiphosphatase; Membrane protein; (*D. discoideum*)

1. Introduction

The structures of the *N*-linked oligosaccharides of *Dictyostelium discoideum* glycoproteins [1] are different from those of vertebrate glycoproteins. The complex oligosaccharides, containing branch GlcNAc, Gal and sialic acid, typical of vertebrate glycoproteins, have never been discovered in *Dictyostelium discoideum*. Instead, 6-phosphomethylated mannosyl- [2], 6-sulfated mannosyl- [3] and β 1 → 4 linked intersecting and bisecting *N*-acetylglucosaminyl-residues are joined to high-mannose backbones [4].

The corresponding sulfotransferase [5], methyltransferase [6] and intersecting GlcNAc transferase (IGTase) [7] activities have been detected and partially characterized. These activities are assumed to be concentrated in the Golgi apparatus. However, with the exception of the NDPase activity reported in this paper, distinguishing Golgi marker enzymes, such as galactosyltransferase and sialyltransferase have not been detected in the organism. Furthermore, no report of immuno-localization of any of these enzymes in any defined structure in electron micrographs and no bona fide marker for *Dictyostelium discoideum* Golgi apparatus is now available. Such information would be useful because the enzymes which catalyze these post-translational modifications are developmentally regulated [8]. A better characterization of the Golgi complex of *Dictyostelium discoideum* is relevant since it might be an excellent model for the study of effects of developmental programs on organelle function.

We have focussed our attention on IGTase. This en-

Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; Man_3-Me, methyl *O*-α-D-mannopyranosyl-(1 → 6)-[*O*-α-D-mannopyranosyl-(1 → 3)]-α-D-mannopyranoside; ACES, 2-[(2-amino-2-oxoethyl)amino]-ethanesulfonic acid; IGTase, UDPGlcNAc:oligosaccharide β-*N*-acetylglucosaminyltransferase; NDPase, nucleotidyl-diphosphatase; Con A, concanavalin A.

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SSDI 0005-2736(95)00298-7

zyme has been shown to be developmentally regulated and vesicle associated. Here we show it is an intrinsic, intravesicular, latent activity which co-localizes with NDPase in vesicles with a density similar to that of rat liver Golgi. We further show that *Dictyostelium discoideum* glycoproteins, destined either for secretion or temporary storage in lysosomes, are modified by IGTase during their transit through the cell. These findings provide evidence that IGTase is a Golgi membrane protein.

2. Materials and methods

2.1. Chemicals

The following radioactive compounds were purchased from DuPont-New England Nuclear (Boston, MA): UDP[U-^{14}C]galactose, 300 mCi/mmol; UDP[4,5^{3}H] galactose, 32.1 Ci/mmol; UDP[6^{3}H]GlcNAc, 36.5 Ci/mmol and [^{35}S]PAPS, 2.03 Ci/mmol. Nucleotides, nucleotide derivatives and galactosyltransferase (EC 2.4.1.22) from bovine milk were purchased from Sigma Chemical Co. (St. Louis, MO). Peptide: *N*-glycanase F (EC 3.5.1.52) was purchased from New England Biolabs (Beverly, MA). Man_3Me was from Pierce (Rockford, IL) and Accurate Chemical (Westbury, NY).

2.2. Subcellular fractionation

Exponentially growing *D. discoideum* cells of the axenic strain, AX2, were harvested by low speed centrifugation and resuspended in 0.25 M sucrose. All sucrose solutions were made in TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) and supplemented with 0.1% (v/v) of a protease inhibitor mixture consisting of 2 mg/ml each of leupeptin, aprotinin and pepstatin A. The following steps were performed at 4°C. Cells were disrupted, by nitrogen cavitation at 500 psi, for 15 min, followed by 20 strokes in a tight-fitting teflon-glass homogenizer. Nuclei and unbroken cells were pelleted by centrifugation at $1000 \times g$ for 5 min. The post-nuclear supernatant was processed as described [9] to obtain a crude membrane fraction. Approx. 80% of this fraction (usually 5 ml) was made 0.25 M in sucrose concentration, loaded on top of a discontinuous sucrose gradient (consisting of 8 ml each of 1.1 M and 0.8 M sucrose) and centrifuged at 27000 rpm for 2.5 h in a SW28 rotor. Material in the pellet, the 0.8/1.1 M or the 0.25/0.8 M interface was collected, diluted with 0.25 M sucrose and reisolated at $100000 \times g$ for 1 h. Pellets were surface washed and resuspended in a suitable volume of 0.25 M sucrose, 1 mM $MgCl_2$, 20 mM ACES, pH 6.5.

2.3. Assays

The following enzyme activities were assayed as described: glucose-6-phosphatase [11], 5'-nucleotidase [11], succinate-cytochrome *c* reductase [11], sulfotransferase [5,12], NDPase [13] and β-hexosaminidase [14]. The IGTase assay was that of Sharkey and Kornfeld [7] except for the following modifications: aliquots of fractions (0.05 to 0.5 mg of protein) were incubated, for 15 min, in a 40 μl reaction mixture, containing 0.25 M sucrose, 20 mM ACES, pH 6.5, 10 mM $MnCl_2$, 2 mM ATP, 0.1 mM UDP-GlcNAc plus 0.2 μCi of UDP-[6-^{3}H]GlcNAc, 36.5 Ci/mmol, and 0.1% Triton X-100, in the presence or absence of 2 mM Man_3-Me (the oligosaccharide acceptor). Since maximum activity was at 37°C, the standard reaction was carried out at 35°C. High sensitivity was achieved by use of high specific activity substrate. To do so, while maintaining the quantity of radioactivity within an operative range, UDP-GlcNAc at 8% of the apparent K_m for the nucleotide sugar was used. The reaction was stopped by adding 0.16 ml of 20 mM EDTA column of AG 1-X2 (Cl^- form) anionic exchange resin. The neutral fraction was quantitatively recovered by washing with 2 ml of water. Radioactivity was measured by liquid scintillation counting and the difference between product generated in the presence and absence of acceptor was considered due to the transferase activity (see below).

2.4. Concanavalin A-Sepharose chromatography

Neutral fraction samples were loaded on 1 ml columns of Con A-Sepharose equilibrated in Con A buffer (1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5), washed exhaustively with the same buffer and eluted with 10 mM α-methylmannoside. The resin was regenerated by washing with 10 bed volumes each of 5 M urea; 10 mM glucose in 1 M sodium citrate, pH 4.5; and 1 M Tris base. Other samples from the same fraction were analyzed by size exclusion using a 1.5×80 cm Sephadex G-10. The column was equilibrated and fractions were eluted with 50 mM NH_4HCO_3. Chromatography was performed at a flow rate of 15 ml per h.

2.5. Synthesis and purification of GlcNAc-Man_3-Me

The tetrasaccharide was synthesized essentially as in the IGTase standard assay, with the exception that the concentration of UDP-GlcNAc and Man_3-Me was increased to 6 mM each, and the incubation time was 60 min. The resulting neutral fraction was separated from remaining Man_3-Me and free GlcNAc by Con A-Sepharose chromatography, and was desalted using Sephadex G-10. The yield was about 5 nmol per reaction.

2.6. Purification of β-hexosaminidase

A partially purified fraction of β-hexosaminidase was obtained from *D. discoideum* spent medium as described [14], with the exception that Sephacryl S-300 was used instead of Sephadex G-200. This preparation was purified 60- to 80-fold by: (1) hydrophobic interaction on a

phenyl-Sepharose CL-4B column (0.9 × 15 cm), equilibrated with buffer A (2 M $(NH_4)_2SO_4$, 10 mM ACES, pH 6.5) and eluted with a 200 ml gradient buffer which started at 100% buffer A and ended with 100% buffer B (10 mM ACES, pH 6.5). (2) Immuno-affinity chromatography employing anti β-hexosaminidase antibodies immobilized on a glutaraldehyde activated Acti-Disk cartridge (FMC Bio products, Pine Brook, NJ) as recommended by the manufacturer. The enzyme was eluted with 0.1 M glycine-HCl, pH 2.5 and immediately neutralized with Tris base.

2.7. Other procedures

Protein was measured by the bicinchoninic acid method (Pierce, Rockford, IL). The following procedures were performed as described in references: labeling of fractions with endogenous GlcNAc transferase [15] and exogenous galactosyl-transferase [15], analysis of the protein oligosaccharide linkage and removal of extrinsic membrane proteins and content [15]; phase separation of integral membrane proteins in Triton X-114 [16], detergent removal with Extracti-Gel D (Pierce, Rockford, IL); SDS-PAGE in the Tricine buffer system, Western blotting and autoradiography [17].

3. Results

3.1. An assay for rapid multiple analyses of Dictyostelium discoideum intersecting transferase

The IGTase assay described here was designed to avoid the time consuming separation of neutral products on Con A-Sepharose, which is part of the published assay [7]. Macromolecular acceptors we tried which would have been easily separated from other products by acid precipitation (soybean agglutinin, porcine thyroglobulin and ribonuclease) were poor substrates. However, subtraction of a substrate-lacking control from experimental values with low molecular weight acceptors, left a value which was totally determined by IGTase activity. This is shown below for Man_3-Me, the assay substrate we chose (see Section 4).

The 0.8/1.1 M fraction of *Dictyostelium discoideum* membranes was incubated with UDP-[^{3}H]GlcNAc, in the presence or absence of this substrate, as described in Section 2. After the incubations were terminated, the neutral molecules were separated by anion exchange chromatography and analyzed on Con A-Sepharose columns. When substrate was absent, a single radioactive peak was observed. It eluted in the loading buffer with authentic GlcNAc. When substrate was present, a second peak was observed (Fig. 1). In many tests like the one shown in the figure, the amount of radioactivity in this peak equalled the difference between total neutral products generated in the presence of substrate and that generated in its absence. This peak eluted from the column at 22°C and at 10 mM

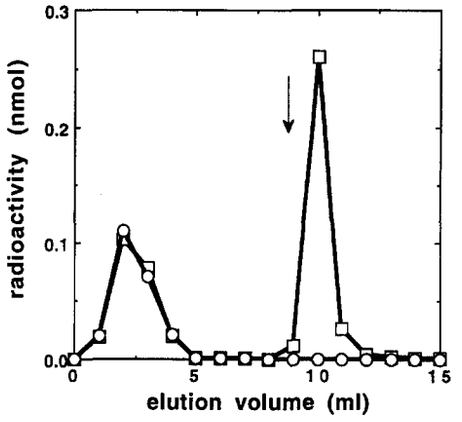

Fig. 1. Con-A Sepharose chromatography of reaction products. UDP-[^{3}H]GlcNac was incubated with 0.1 mg of protein from the 0.8/1.1 M interface fraction under standard assay conditions, in the presence (open square) or absence (open circle) of Man_3-Me. Aliquots were removed for direct measurement of neutral reaction products. From these measurements, IGTase activity was calculated to be 173.5 pmol/min/mg, by difference. The remaining 90% of each reaction was analyzed by Con-A Sepharose affinity chromatography as described in Section 2. Radioactivity retained in the column was measured. And the IGTase activity was calculated to be 171.1 pmol/min/mg. The arrow indicates the start of elution with 10 mM α-methylmannoside.

α-methylmannoside. By contrast, Man_3-Me eluted at 60°C with 100 mM α-methylmannoside [7]. These findings were in consonance with the assumption that the peak was GlcNAc-Man_3-Me.

In another set of experiments, the neutral fraction was obtained as described in Fig. 1 but it was analyzed by size exclusion chromatography (Fig. 2). In the absence of substrate, one peak eluted at the position of monosaccharides. In the presence of substrate, two peaks were de-

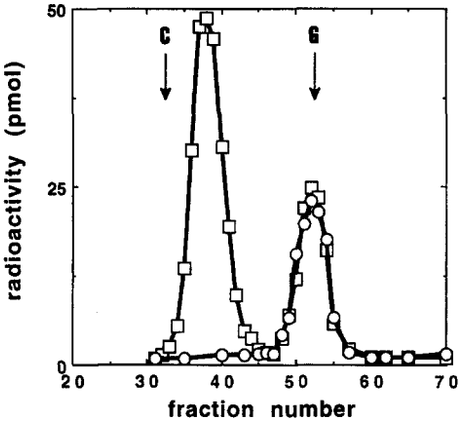

Fig. 2. Sephadex G-10 chromatography. IGTase was measured as described in Fig. 1. with (open square) and without (open circle) Man_3-Me for 15 min, 90% of each sample was loaded on a Sephadex G-10 column, 2 ml fractions were collected and the void volume was determined using cytochrome *c*. By this procedure the IGTase activity was calculated as 176.2 pmol/min/mg. It was calculated as 177.6 pmol/min/mg by difference. Arrows indicate the elution volume of (C) cytochrome *c* and (G) GlcNAc.

tected. The larger one eluted where tetrasaccharides are expected [18]. As in Fig. 1, the quantity of radioactivity in the substrate dependant peak was equal to the difference between the total neutral radioactivity produced in the presence of Man_3-Me and that in its absence.

With Man_3Me as substrate, IGTase activity was a linear function of time for at least 20 min, both in the crude membrane fraction and in the 0.8/1.1 M fraction (Fig. 3A). IGTase activity was also a linear function of the amount of protein added, from 50 to 400 μg (Fig. 3B). Even when 400 μg of proteins were incubated for 20 min more than half of the UDP-GlcNAc remained (as measured by HPLC) [19]. As previously described, neither Mg^{2+} nor Ca^{2+} could substitute for Mn^{2+} in the reaction. Either in the absence of added Mn^{2+} or in the presence of 20 mM EDTA, the activity of the enzyme was less than 1% that of the control (data not shown). These properties are identical to those observed with the published assay [7].

To optimize activity, we measured the effect of temperature on IGTase activity. The optimal temperature was 37°C. An Arrhenius plot of the activity data revealed a sharp change in slope with a thermal transition at 16°C (Fig. 4). The activation energy was 24.3 kcal/mol between 0°C and 16°C, and 4.6 kcal/mol for higher temperatures. This suggested that the enzyme might be an intrinsic membrane protein (Shinitzky, M., personal communication), like others which exhibit this transition, such as the rat liver Golgi sugar-nucleotide translocators (Capasso, J.M., unpublished data).

3.2. *IGTase and NDPase are vesicular enzymes which co-localize in the same subcellular fractions of Dictyostelium discoideum*

To localize IGTase, standard fractionation procedures were employed. Because we thought IGTase might be a Golgi enzyme, we also measured NDPase activity. This enzyme was shown to be a Golgi membrane marker in several other organisms, including yeast [20] and mammals [13,21]. As shown in Table 1, 96% of the IGTase activity was recovered in the post-nuclear supernatant and about

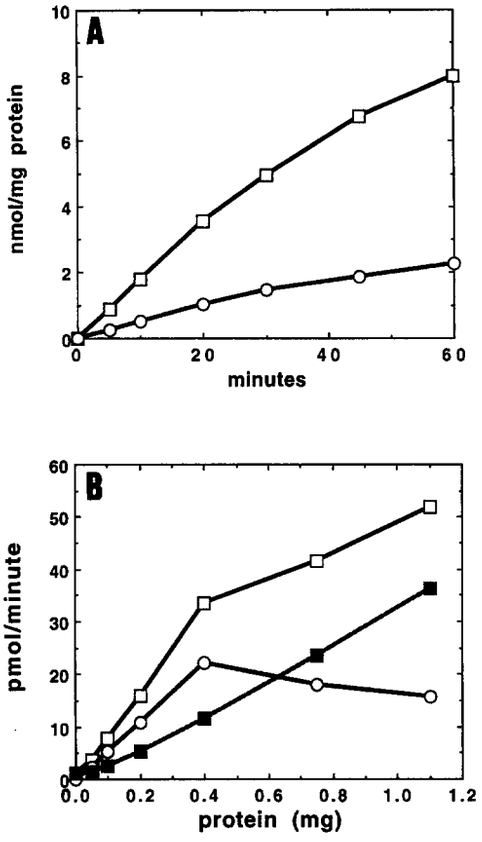

Fig. 3. (A) Effect of time of incubation on amount of IGTase product generated. IGTase activity was measured as described in Section 2 with 50 μg of 0.8/1.1 M interface fraction (open square) or 200 μg of crude membrane fraction (open circle) proteins. The amount of product was calculated by subtracting the amount of radioactivity in the absence of Man_3-Me from that in its presence. Results are averages of three separate determinations. (B) Effect of protein concentration on amount of IGTase product. IGTase activity was measured as described for 20 min with indicated amounts of crude membrane proteins, either in the presence (open square) or in the absence (closed square) of acceptor. The amount of product (open circle) was calculated as in A. Results are the average of three separate determinations.

50% of the activity, purified 3.4-fold from the homogenate (Table 1), was recovered in the crude membrane fraction. When crude membranes were subjected to discontinuous

Table 1
Sub-cellular distribution of IGTase activity

Sub-cellular fraction	Specific activity (pmol/m/mg)	Percentage of activity (%)	Enrichment
Homogenate	17.2 ± 2.0	–	–
Post nuclear supernatant	20.7 ± 2.5	96.0 ± 5.9	1.2 ± 0.1
Crude membranes	47.6 ± 2.8	50.5 ± 5.3	3.4 ± 0.1
0.25/0.8 M interface	44.7 ± 3.5	1.9 ± 0.4	2.4 ± 0.3
0.8/1.1 M interface	187.0 ± 3.9	16.2 ± 0.7	11.1 ± 0.8
Pellet	1.9 ± 0.7	1.3 ± 0.3	0.2 ± 0.1

IGTase was measured in the indicated sub-cellular fractions. These were prepared as described in Section 2. Specific activity is expressed as pmol/m/mg protein. Enrichment is the specific activity of each fraction/specific activity of the homogenate. All values are the average ± the standard deviation of 8 different experiments. Glucose-6-phosphatase was enriched 4.2-fold in the pellet and was essentially absent from the 0.8/1.1 M interface.

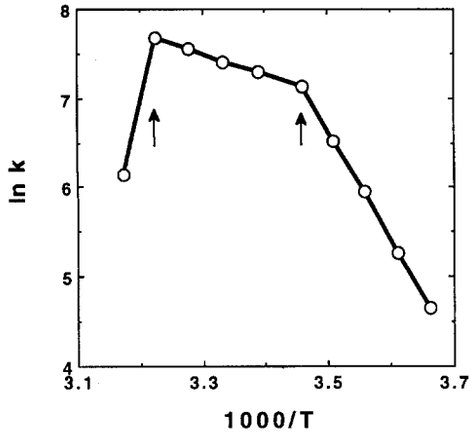

Fig. 4. Arrhenius plot of the effect of temperature on IGTase reaction rates. IGTase was measured in aliquots of the 0.8/1.1 M fraction. Each contained 0.1 mg of protein. The concentration of substrate was 6 mM UDP-GlcNAc (5 mCi/mmol) and the product was measured after purification by Con A chromatography. Before the reaction was started all its components were equilibrated at the appropriate temperature in a thermocycler. Plotted values are the average of two determinations. IGTase activity at 37°C was 214.3 pmol/min. Arrows mark the optimal temperature, 37°C and the thermal transition, 16°C.

sucrose gradient centrifugation, IGTase activity was greatly enriched in the fraction sedimenting at the interface between 1.1 M and 0.8 M sucrose. It was dramatically reduced in the pellet.

As shown in Table 2, the sub-cellular distribution of NDPase activity, measured with 5′-UDP as its substrate, was the same as that of IGTase. NDPase specific activity also was enriched 8–10-fold in the 0.8/1.1 M interface fraction and was lower in the pellet than in crude membranes. Similar results were obtained using either 5′-ADP or 5′-CDP as substrate (data not shown). By contrast, glucose-6-phosphatase, an endoplasmic reticulum marker, β-hexosaminidase, a lysosomal marker and succinate-cytochrome *c* reductase, a mitochondria marker, showed a 4.2-, 3.9- and 3-fold enrichment in the pellet, respectively, but a 4- to 5-fold depletion in the 0.8/1.1 M interface fraction. The plasma membrane marker, 5′-nucleotidase, showed less than a 2-fold enrichment in that fraction.

Table 3
Nature of the interaction of IGTase and NDPase with membranes

Treatment	IGTase activity (%)	NDPase activity (%)	Sialyl-Tase activity (%)
Panel A			
Carbonate pellet	85.4	83.2	89.3
Carbonate supernatant	2.9	4.6	3.7
Panel B			
Hydrophobic fraction	70.2	76.7	72.0
Hydrophilic fraction	5.2	3.9	4.8

Aliquots of the 0.8/1.1 M fraction from *D. discoideum* and Golgi from rat liver (obtained as described [3]), each containing 1 mg of protein were fractionated as described in Section 2 with sodium carbonate (Panel A) or Triton X-114 (Panel B). *D. discoideum* IGTase and NDPase activities were measured as described in Section 2. Rat liver Golgi sialyltransferase was measured as described [20]. Results are expressed as percent of the initial activity and are the average of three independent experiments. Initial activities were: IGTase 185.3 pmol/m; NDPase 25.0 nmol/m and sialyltransferase 10.1 nmol/m.

The 0.8/1.1 M interface fraction also contains endogenous macromolecular acceptors of GlcNAc. Aliquots of the 0.8/1.1 M interface were incubated as described in Methods, with radioactive UDP-[^{3}H]GlcNAc. When the incubation products were analyzed by SDS gel electrophoresis and fluorography, a complex pattern of radiolabeled proteins was detected. Most of the [^{3}H]GlcNAc was attached to *N*-glycosides as indicated by its removal by peptide: *N*-glycanase F treatment (data not shown).

3.3. IGTase and NDPase are intrinsic membrane proteins

Intrinsic proteins are often resistant to alkaline extraction. Vesicles from the 0.8/1.1 M interface were washed with Na_2CO_3-EDTA, a procedure known to rupture membrane vesicles and remove their vesicular content and extrinsic membrane proteins [15]. About 85% of the initial

Table 2
Sub-cellular distribution of NDPase activity

Sub-cellular fraction	Specific activity (nmol/m/mg)	Percentage of activity (%)	Enrichment
Homogenate	2.2 ± 0.2	–	–
Post nuclear supernatant	2.9 ± 0.4	93.2 ± 7.7	1.3 ± 0.2
Crude membranes	8.4 ± 0.7	48.7 ± 6.4	3.9 ± 0.1
0.25/0.8 M interface	4.9 ± 0.5	1.2 ± 0.3	2.3 ± 0.3
0.8/1.1 M interface	21.4 ± 1.5	13.9 ± 0.7	9.9 ± 0.8
Pellet	6.5 ± 0.3	23.6 ± 3.0	3.0 ± 0.2

NDPase was measured, using 5′-UDP as substrate, in the indicated sub-cellular fractions obtained as described in Section 2. Specific activity is expressed in nmol/m/mg protein. The enrichment is the specific activity of each fraction/specific activity of the homogenate. All values are the average ± the standard deviation of 4 different experiments.

IGTase and NDPase activities was recovered in the carbonate washed membrane pellet. Less than 5% was detected in the supernatant fraction, even after extensive desalting and concentration (Table 3, panel A).

Intrinsic membrane proteins are often rich in hydrophobic domains. Consequently they partition in (Triton X-114)-rich phases. The distribution of IGTase and NDPase was evaluated by this test. A portion of the vesicle preparation was solubilized in 1% Triton X-114, in the cold, then phase separation was induced by raising the temperature of the mixture to 30°C. A small, detergent rich, hydrophobic fraction and a larger, detergent depleted, hydrophilic fraction were obtained. More than 70% of the initial IGTase and NDPase activities was recovered in the hydrophobic fraction and about 5% in the hydrophilic one (Table 3, panel B). These characteristics of IGTase and NDPase are identical to those observed for the rat liver Golgi sialyltransferase (Table 3).

3.4. IGTase and NDPase are intralumenal membrane proteins

We attempted to determine the orientation of the active sites of the enzymes in the vesicles. Neither IGTase nor NDPase activity was fully available to the substrate until the vesicles were disrupted by 0.1% Triton X-100. The degree of latency (78% to 85% of each activity) was dependent on the batch of vesicles used. In intact vesicles, intralumenal enzymes, such as rat liver Golgi sialyltransferase [11], are resistant to certain Pronase digestion procedures. As shown in Table 4, greater than 80% of the original IGTase and NDPase activities were recovered when aliquots of the 0.8/1.1 M fraction were digested with pronase at fixed protease/protein ratios, without added detergent. When the digestion was carried out in the presence of 0.1% Triton X-100, less than 10% of the activities were recovered, even at the lowest protease/protein ratio.

Table 4
Effect of pronase treatment of vesicles on IGTase activity

Pronase/ protein ratio	IGTase activity (%)	NDPase activity (%)	Sialyltransferase activity (%)
1:100	83.3	82.7	94.9
1:40	79.2	83.6	93.1
1:100 plus 0.1% Triton	7.5	10.1	6.4

Aliquots of the 0.8/1.1 M fraction from *D. discoideum* and Golgi apparatus from rat liver, each containing 1 mg of protein were digested with different amount of Pronase in STKM buffer at room temperature for 30 m, the reaction was stopped with a mixture of proteinase inhibitors and vesicles were isolated immediately by centrifugation through a cushion of 0.5 M sucrose in TKM buffer, pellets were surface washed, resuspended, and IGTase, NDPase and sialyltransferase activities were measured as in Table 3. Results are expressed as percentage of the initial activity and are the average of three independent experiments. Initial activities were as in Table 3.

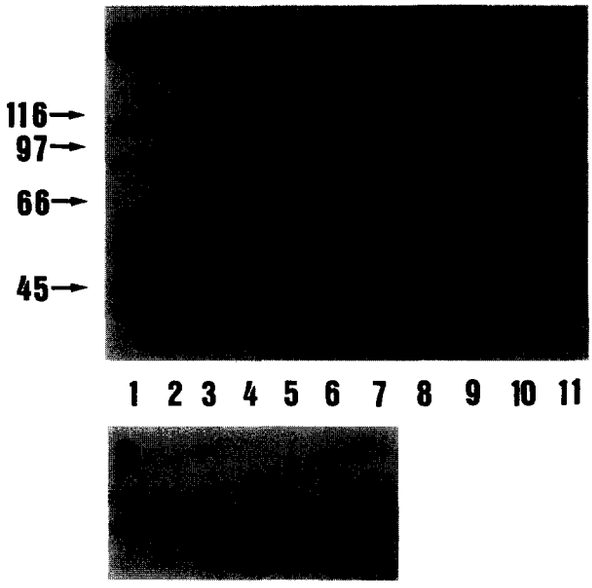

Fig. 5. Evidence that endogenous proteins from *Dictyostelium discoideum* are modified by IGTase. (A) SDS-PAGE autoradiogram of [^{3}H]Gal labeled glycoproteins. Aliquots containing glycoproteins purified to near homogeneity from spent medium were labeled with UDP-[^{3}H]Gal and bovine milk galactosyltransferase as described in Section 2. Lanes 1 to 7, β-hexosaminidase; lanes 8 and 9, Hex 22-Invertase; lanes 10 and 11, Hex 70-Invertase. Then samples were either immediately prepared for electrophoresis (lane 1), or denatured and incubated for 24 h at 37°C without (lanes 2, 9 and 11) or with (lanes 7, 8 and 10) N-glycanase F. Lanes 3 to 6 were incubated with the same glycanase for 15, 30, 60 and 120 min, respectively. (B) Coomassie blue staining of the same gel showing lanes 1–7. Arrows indicate the position of the standard molecular mass markers in kDa.

3.5. Endogenous glycoproteins from Dictyostelium discoideum are modified by IGTase

The transfer of [^{14}C]Gal to macromolecules, catalyzed by galactosyltransferase, was used to observe the presence of terminal GlcNAc, the product of IGTase activity. The intersecting GlcNAc is accessible to the transferase. This was shown as follows: GlcNAc-Man_3-Me was synthesized and purified as described in Methods. It was then treated with UDP-[^{14}C]Gal and galactosyltransferase. The product was analyzed by size exclusion chromatography on Sephadex G-10, as in Fig. 2. The tetrasaccharide was an excellent acceptor of galactose. When 1 mM of GlcNAc-Man_3-Me was used, 1360 pmol of [^{14}C]Gal were detected in the peak migrating near the exclusion volume. In contrast, none was detected there when either Man_3-Me or α-methylmannoside, possible contaminants of the preparation, was used as the acceptor.

β-Hexosaminidase, a lysosomal glycoprotein isolated from the secretions of stationary phase cultures behaved in a similar fashion. An aliquot of the highly purified enzyme from *Dictyostelium discoideum* (nearly homogenous by the criterion of SDS-polyacrylamide gel electrophoresis followed by silver staining) was labeled in the same way. As shown in Fig. 5, lane 1, both forms of β-hexosamini-

dase were labeled. After treatment with *N*-glycanase, most of the radioactivity was removed after 15 min. of incubation (lane 3) and the pattern did not change, even after as much as 24 h of incubation (lane 3–7). The removal of the radioactivity was not due to endogenous activities since a 24 h incubation without the enzyme did not change the labeling pattern (compare lanes 1 and 2). Loss of radioactivity was coincident with a shift in the mobility of both forms of β-hexosaminidase (lanes 2 and 3 of panel B), consistent with the loss of *N*-linked oligosaccharides. The same results were obtained with either Hex22-Invertase or Hex70-Invertase, both fusion proteins [10] (lanes 8–11).

4. Discussion

These studies have led to the development of better assays for IGTase and for the products of the IGTase catalyzed reaction. The assays were used to localize the enzyme. We present evidence that the enzyme is a vesicular integral membrane protein whose catalytic site faces the lumen. We also present several lines of evidence which indicate that the activity is localized in the Golgi apparatus. These findings are directing us to further studies which may help elucidate the mechanisms of developmental regulation of the organelle.

Features of the IGTase assay described here include its relative speed, sensitivity and lack of ambiguity. Furthermore, its simplicity makes multiple simultaneous assays feasible. The procedures are reminiscent of the standard galactosyltransferase assay [22]. A rate of 0.1 pmol/min can easily be detected, under standard conditions. We chose Man_3-Me as substrate because: (1) the position to which GlnNAc attaches to this acceptor is unambiguously intersecting, mimicking the in vivo situation. This is not the case for oligosaccharides with more mannose, to which GlcNAc might also be attached in the bisecting position. (2) Of all oligosaccharides tested, the enzyme has the highest V_{max} for Man_3-Me. (3) Man_3-Me is readily available from commercial sources and it is economical.

The assay of IGTase products is based on the specific transfer of Gal from UDP-Gal to terminal GlcNAc glycosides, catalyzed by galactosyltransferase. Loss of macromolecular galactosyl-residues, subsequent to treatment with protein *N*-glycanase indicates that the transfer is to *N*-glycosides. In *D. discoideum* all the branch GlcNAc on *N*-glycosides is derived from the action of IGTase.

The evidence that IGTase is an intrinsic lumenal vesicle membrane protein begins with the finding that 50% is recovered in a crude membrane fraction (Table 1 and [7]). Most of this is resistant to alkaline extraction (Table 3), and most is partitioned into a Triton X-114 rich hydrophobic phase (Table 3). Furthermore, the Arrhenius plot for the activity exhibits a break at 16°C (Fig. 4). Each of these results is an expected property of intrinsic membrane proteins [15,16]. The findings that the activity is latent and unavailable to Pronase in the absence of detergent but available to substrate and sensitive to Pronase in the presence of detergent are now considered standard tests for the lumenal topology of the active site of the enzyme [11–13]. This differentiates IGTase activity from endoplasmic reticulum GlcNAc transferase, whose orientation is toward the cytosol [23].

The easy separation of the transferase from glucose-6-phosphatase by centrifugation in discontinuous sucrose gradients also indicates IGTase is not an endoplasmic reticulum protein. Its co-localization and co-enrichment with NDPase in the same fractions by two different sedimentation procedures, favors the idea that it is retained in the Golgi apparatus. NDPase is a known Golgi marker in other organisms playing an important part in the membrane transport system of nucleotide-sugars, 5′-ATP and PAPS [24]. This proposal is bolstered by the following observations: (1) the density of the most enriched fraction of the enzyme is similar to that of rat liver Golgi [25]; (2) another supposed Golgi activity, sulfotransferase, is also enriched in this fraction [5], as are early forms of lysosomal enzymes [26] and endogenous substrates for the transferase.

Further support comes from the results in Fig. 5 that show proteins which have passed through the lysosomes (β-hexosaminidase), prelysosomal compartments (Hex70-Invertase) or secretory vesicles (Hex22-Invertase) [10], each carry moieties which are the result of IGTase action. The simplest explanation for this is that they all pass through a compartment which holds IGTase before they are sorted. Since the endoplasmic reticulum is excluded by the results described above, that compartment is most likely the Golgi apparatus. However, these studies do not exclude a more diffuse localization in Golgi, transit vesicles and prelysosomal compartments.

The strongest evidence for Golgi retention of IGTase will come from immuno-localization of the enzyme with electron micrography. Since the *D. discoideum* Golgi apparatus may be an excellent model of a developmentally regulated organelle, it is important to pursue this effort in the future.

Acknowledgements

We are indebted to Lic. Diana M. Korchak for the critical reading of the manuscript and helpful suggestions. We thank Jo Ann Etters for her skillful secretarial assistance. This work was supported in part by a Grant from NSF #MCB-9304439.

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