Abstract  A fucosyltransferase activity has been detected using lacto-N-biose I as acceptor in the lower eukaryote Dictyostelium discoideum. This transferase requires divalent cations and is inhibited by N-ethylmaleimide and detergent treatment. Apparent calculated $K_m$ values for GDP-Fuc and lacto-N-biose I are 1.27 $\mu$M and 2.80 mM, respectively. The activity is quantitatively recovered in the supernatant after centrifugation at 100 000 $\times g$ for 1 h. The reaction product, as determined by gel permeation chromatography, sensitivity to fucosidases, and analysis of partially methylated derivatives, is Fuc(1→2)Gal(1→3)GlcNAc (H type 1 trisaccharide).

Key words: Glycosyltransferase; Cytosol; Carbohydrate antigen; Permethylation; (Dictyostelium discoideum)

1. Introduction

Glycosyltransferases have been mostly reported as membrane-bound enzymes involved in the biosynthesis of the saccharide chains of glycoproteins and glycolipids [1]. Glycosyltransferases found in biological fluids reach the extracellular compartment from the Golgi lumen through the secretory pathway, and proteolytic cleavage eventually makes them refered to as the homogenate. It was spun at 2000 $\times g$ for 6 min, at 4°C, inhibited by N-ethylmaleimide and detergent treatment. Appar...
source. Fuc-TIII activity was determined in a reaction mixture con- 
taining, in a final volume of 0.3 mL, 0.05 M Tris-HCl buffer, pH 7.4, 10 mM Me2Cp, 0.5 mg/ml Triton X-100, 40 μM donor GDP-[3H]Fuc (spec. act. 100 mCi/mmol), 10 mM acceptor lacto-N-biose I, and 0.25 mg/ml enzyme protein, corresponding to about 4 × 10^3 cells per reaction. Incubation was performed at 37°C for 60 min. H type 1 trisaccharide was prepared in a reaction mixture containing, in a final volume of 0.1 M Tris- HCl buffer, pH 7.4, 6.5 μg M, 0.5 mg/ml Triton X-100, 18 μM donor GDP-[3H]Fuc (spec. act. 500 mCi/mmol), 40 mM acceptor lacto-N-biose I, and 6.4 mg/ml enzyme protein, corresponding to about 5 × 10^3 cells. Incubation was carried out at 37°C for 90 min. Inhibition studies with NEM were carried out as reported by Stroup et al. [10]. Reaction product was assayed by Dowex 1 × 8 anion exchange columns and radioactivity determined by liquid scintillation counting as reported [8]. Blanks were prepared by omitting the acceptor in the reaction mixture and the incorporation values subtracted for enzyme activity calculations.

2.4. Reaction product characterization

Quenched reaction mixtures were thawed, pooled, chromatographed on Dowex columns as above, and lyophilized. The obtained material was resuspended in water and 0.1 ml aliquots (corresponding to about 20 000 000 pmol of product) were loaded on a Biogel P2 (Bio Rad) column (0.7 cm × 50 cm) prepared and equilibrated in 0.1 M Tris-HCl buffer, pH 8.5, and radioactivity was determined in the elution fraction, while no activity is detectable in the membranes.


taining, in a final volume of 0.3 mL, 0.05 M Tris- HCl buffer, pH 7.4, 10 mM MgCl2, 10 mM Fuc, 5 mM Me2Cp, and allowed to react at 23°C for additional 24 h.

3. Results

3.1. Detection and subcellular distribution of fucosyltransferase activity toward lacto-N-biose I in Dictyostelium

In the presence of lacto-N-biose I, a reproducible acceptor- 
dependent incorporation of radioactivity into the neutral com- 
ponents unrestrained by Dowex columns is measurable using cell homogenate, postnuclear and cytosolic fractions. The distri- 
bution of protein and Dxt1.2FucT activity in subcellular 
fractio
determined in a reaction mixture containing, in a final volume of 0.3 mL, 0.05 M Tris-HCl buffer, pH 7.4, 10 mM Me2Cp, 10 mM Fuc, 5 mM ATP, 0.5 mg/ml Triton X-100, 75 μM unlabelled GDP-Fuc, 0.12 mM GlcNAc-3H]lacto-N-biose I (spec. act. 0.5 Ci/ mmol) and 40 mg/ml Fuc-TIII protein, as transfected-COS-7 cell homogenate (see Section 2.3). Incubation was for 22 h at 37°C. Tri- 
saccharide products were purified by Dowex and Biogel P2 chroma-
tography as described for the (Fuc-3H]-labelled trisaccharides. Indi-
vidual column fractions were lyophilized, resuspended in 20 μl of 
water, and analyzed by HPTLC.
3.2. Characterization of the enzymatic activity

Values are the activity values expressed as pmol/mg protein per h transferred Fuc. by HPTLC, and comigrate with reference standard Fuc (not according to the formula: \[ S/v = \frac{1}{V_{\text{max}}} \times \frac{S}{K_{\text{m}}} \], where velocity (v) = the activity values expressed as pmol/mg protein per h transferred Fuc.

![Graph](Fig. 1. Kinetic characterization of Dct1,2FucT. Enzyme activity measurements were carried out as described under Section 2. The effect of donor GDP-Fuc (upper panel) and acceptor lacto-N-biose I (lower panel) is presented. Blank values obtained in parallel reactions lacking the acceptor were subtracted from each point. Activity data (○) represent duplicate assays. The right scale (●) shows the transposition in the Hanes-Woolf plot of the activity values according to the formula: \[ S/v = \frac{1}{V_{\text{max}}} \times \frac{S}{K_{\text{m}}} \].)

3.3. Purification and fucosidase treatment of the reaction product

The reaction products with unlabelled lacto-N-biose I as acceptor and GDP-[3H]Fuc as donor of Dct1,2FucT, Hct1,2FucT (H type 1 trisaccharide) and Fuc-TIII (Lewis a trisaccharide), obtained by Dowex chromatography, were freeze-dried and subjected to gel permeation chromatography on Biogel P2. Hct1,2FucT reaction provides two peaks which represent over 95% of the loaded radioactivity. The first peak, representing 91% of the recovered radioactivity, is assumed as the reference H type 1 trisaccharide; the second peak is eluted at the size of monosaccharides. Fuc-TIII reaction provides two peaks representing 91% of loaded radioactivity. Both peaks correspond to those obtained with Hct1,2FucT and Fuc-TIII, but in this case the first peak, eluted at the same size of H type 1 and Lewis a trisaccharides, represents 71% of the recovered radioactivity. H type 1 trisaccharide, Lewis a trisaccharide, and the corresponding peak obtained with the Dct1,2FucT product, as well as the minor peaks eluted in the monosaccharide area, provide single spots when analyzed by HPTLC. Spots derived from the minor peaks appear identical by HPTLC, and comigrate with reference standard Fuc (not shown). The spot derived from the Dct1,2FucT major peak comigrates with H type 1 trisaccharide, moves faster than the Lewis a trisaccharide, and is affected by bovine epididymis α-fucosidase but not by almond meal α-fucosidase (Fig. 2).

Under the conditions used (see Section 2), the H type 1 trisaccharide is also sensitive to bovine epididymis α-fucosidase, and insensitive to the almond meal enzyme, while the Lewis a trisaccharide is totally sensitive to almond meal α-fucosidase treatment, and almost insensitive to bovine epididymis α-fucosidase (not shown).

![Diagram](Fig. 2. HPTLC analysis and fucosidase treatment of Dct1,2FucT reaction product. Lanes: (1) purified reaction product of Dct1,2FucT; (2) bovine epididymis α-fucosidase treatment of lane 1; (3) almond meal α-fucosidase treatment of lane 1; (4) H type 1 trisaccharide (purified reaction product of Hct1,2FucT); (5) Lewis a trisaccharide (purified reaction product of Fuc-TIII). Reaction product purification and fucosidase treatments were performed as described under Section 2. HPTLC plate was developed using n-propanol/ethyl acetate/water/25% ammonia (6:1:3:1, v/v), as the eluting solvent system; detection was by fluorography. An additional lane, spotted with unlabelled reference standard Fuc, was cut after developing the plate and detected by anisaldehyde spray reagent.)

3.4. Analysis of partially methylated derivatives

The reaction products of Dcd,2FucT with [Gal-3H]lacto-N-biose I or [GlcNAc-3H]lacto-N-biose I as acceptors and unlabelled GDP-Fuc as donor, purified by Dowex chromatography, gel permeation chromatography on Biogel P2, and preparative HPTLC, are chromatographically identical to the corresponding (Fuc-3H)-labelled product. After permethylation, hydrolysis, and analysis by HPTLC in two solvent systems, the [Gal-3H]trisaccharide product of Dcd,2FucT provides a spot moving slower than that obtained with unreacted [Gal-3H]lacto-N-biose I (identified as 2,3,4,6-tetra-O-methylgalactose) and corresponding to reference 3,4,6-tri-O-methylgalactose (Fig. 3). After parallel permethylation, hydrolysis, and analysis by HPTLC, the (GlcNAc-3H)-labelled trisaccharide product of Dcd,2FucT provides a spot comigrating with that obtained with unreacted [GlcNAc-3H]lacto-N-biose I (identified as 4,6-di-O-methylglucosamine), which moves faster than reference mono-O-methylglucosamine (Fig. 3).

4. Discussion

In this paper we present evidence that the fucosyltransferase from the cytosol of Dictyostelium AX2 cells is able to use lacto-N-biose I as acceptor forming the H type 1 trisaccharide product. Optimum assay conditions include neutral pH and 23°C temperature, and divalent cations are absolute requirements for activity detection. Dcd,2FucT activity is quantitatively recovered in a cytosolic fraction, with respect to the homogenate, and is undetectable in membrane fractions. Accordingly, its activity is not stimulated, but instead inhibited, by detergent treatment. In addition it has a very high affinity for GDP-Fuc and is not able to use LacNAc efficiently as substrate. These data strongly suggest that Dcd,2FucT is the same fucosyltransferase as recently purified by West et al. [4]. We also found that Dcd,2FucT is inhibited by NEM, as other fucosyltransferases [10,13]. In this regard, it has been recently suggested that NEM sensitivity is related to specific amino acid sequence in the GDP-Fuc binding domain [14]. Dcd,2FucT activity transfers Fuc only to the galactose moiety of lacto-N-biose I in an α-1 linkage. Upon gel permeation chromatography, the (Fuc-3H)-labelled product was found of the same size of H type I and Lea trisaccharides. In addition, analysis by a rapid and effective HPTLC method indicated that it comigrates with H type 1 trisaccharide and is sensitive to α-fucosidase treatment. In particular, we found that fucose is almost completely cleaved from the Dcd,2FucT product by bovine epididymis α-fucosidase under conditions that leave Lewis a trisaccharide almost unaffected. Treatment with almond meal α-fucosidase determines the opposite effect. Bovine epididymis α-fucosidase is known to cleave α-1 fucosyl residues [15] but its strict specificity is not defined, while almond meal α-fucosidase is considered to have α-1 specificity [16]. In order to establish unambiguously sugar substitution and linkage position, we perform a permethylation study. For this purpose, we prepared enough Dcd,2FucT product radiolabeled either on the Gal or the GlcNAc moiety. The results clearly indicate that only the galactose residue is substituted in the product, while the GlcNAc residue is not. Since the partially methylated galactose obtained comigrates with reference 3,4,6-tri-O-methylgalactose, we conclude that the reaction product of Dcd,2FucT with lacto-N-biose I as acceptor is Fucα1-2Galβ1-3GlcNAc (H type 1 trisaccharide). Dcd,2FucT does not efficiently utilize Gal and β-galactosides different from lacto-N-biose I, which instead are acceptors commonly used by mammalian α1,2fucosyltransferases. Since the putative endogenous substrate of Dcd,2FucT is reported to lack hexosamines [5], we suggest that the Galβ1-3GlcNAc sequence mimics in vitro the FP21 oligosaccharide structure required for enzyme recognition, that is not defined at the present. In this regard, it is interesting to note that the secretor-type α1,2fucosyltransferases prefer type 1 chain acceptors, but can still utilize Gal, phenyl-β-galactoside, and type 2 acceptors, and are not stimulated by divalent cations [17-19]. Our data indicate that Dcd,2FucT is distinct from other α1,2 fucosyltransferases but retain the ability to biosynthesize the H type 1 trisaccharide in vitro, while cytosolic glycosyltransferases reported to date do not share relevant common characteristics with membrane enzymes. Other known cytosolic glycosylations involve the construction of short disaccharide sequences or the addition of single monosaccharide units, which are thought to be mainly regulatory modifications; potential roles include enzyme regulation, protein folding and stability, and intracellular transport [2]. The characteristics of Dcd,2FucT here reported, together with the information available on FP21 [3-5], suggest an even more complex role of such a cytosolic glycosylation pathway in Dictyostelium.

Acknowledgements: The authors wish to thank Dr. Hudson H. Freeze for critical reading of the manuscript, Dr. Maurizia Vailli, Dr. Alberto Passi, Prof. Ruggero Tosti, and Prof. Giuseppe Cetta for the use of their facilities, Antonio Mortara for technical assistance, and Marco Bellaviti for preparation of the artwork. This work was supported by grants of the Italian Ministry of University and Research and of the European Union (ERBCHRXCT930250) to S.B. During the first part of this work M.T. received a fellowship from the University of Turin. M.T. is a researcher at the University of Pavia Medical School II.
References