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Cell-free biosynthesis of erythroglycan in a microsomal fraction from K-562 cells

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Particulate membrane preparations from K-562 [human CML (chronic-myelogenous-leukaemia)-derived] cells catalyse the transfer of [^{3}H]galactose from UDP-[^{3}H]-galactose and [^{3}H]*N*-acetylglucosamine from UDP-[^{3}H]*N*-acetylglucosamine into an endogenous product that on digestion with Pronase yields long-chain glycopeptides (mol.wt. 7000–10000) called 'erythroglycan'. Incorporation of either labelled sugar increased up to 60 min of incubation time. The labelled erythroglycan was isolated by chromatography on Sephadex G-50 and characterized by digestion with endo-β-galactosidase from *Escherichia freundii*, followed by analysis on Bio-Gel P-2 and paper chromatography. This digestion gave the following four products: (1) a disaccharide with the sequence βGlcNAc-βGal; (2) a trisaccharide with the sequence βGal-βGlcNAc-βGal; (3) a larger oligosaccharide containing galactose and *N*-acetylglucosamine; and (4) a putative protein-linkage region.

Asparagine-linked carbohydrate chains occur in at least three major forms in humans. These are: the 'high-mannose' type; the 'complex type' (Kornfeld & Kornfeld, 1976), with a trimannosyl core bearing one lactosamine subunit on one to four sites, terminated with sialic acid; and the 'erythroglycan' (keratan-like) polymers (Jarnefelt *et al.*, 1978; Fukuda *et al.*, 1979; Krusius *et al.*, 1978), with a trimannosyl core bearing two or more polylactosamine chains, which may be very large and branched (Jarnefelt *et al.*, 1978), very large and linear (Fukuda *et al.*, 1979; Krusius *et al.*, 1978), or only a few subunits long (Li *et al.*, 1980). The biosynthetic mechanisms for the high-mannose and complex-type saccharide chains have been extensively studied (for a review, see Parodi & Leloir, 1979), but little is known about the synthesis of erythroglycan, which occurs on relatively few proteins and requires additional enzymes. On erythrocytes the long-chain glycopeptides seem to be principally a substituent of band 3 (Fukuda *et al.*, 1979; Tanner & Boxer, 1972; Gahmberg *et al.*, 1976; Drickamer, 1978) with a small amount evident in the band-4.5 region. Glycophorin, on the same cell, in addition to fifteen *O*-linked chains, bears one *N*-linked chain, which is always of the complex type (Winzler, 1969). K-562 cells (originally from a chronic-myelogenous-leukaemia patient), like erythrocytes, also carry both linear erythroglycan and complex-type chains on their cell-surface proteins (Turco *et al.*, 1980). However, when these cells are infected with vesicular-stomatitis virus, the viral G-protein bears only the complex-type chains (Rush *et al.*, 1981). Thus there is a selective glycosylation of various types of *N*-linked sugar chains, with sialic acid terminating complex chains destined for one protein, whereas another polypeptide may carry only the erythroglycan chains. To begin to answer several questions about the biosynthetic specificity of *N*-linked glycosylation, we have developed a cell-free microsomal system in K-562 cells to examine the additional glycosyltransferases necessary to build a polylactosamine chain on to the *N*-linked 'core' of specific proteins. In the present paper we describe the biosynthesis of erythroglycan-like products from incubation of the microsomal fraction derived from K-562 cells with UDP-[^{3}H]*N*-acetylglucosamine and UDP-[^{3}H]galactose.

Materials and methods

Endo-β-galactosidase, β-*N*-acetylhexosaminidase and β-galactosidase were generously given by Professor Y.-T. Li and Professor S.-C. Li, Delta Regional Primate Research Center, Covington, LA, U.S.A., and Department of Biochemistry, Tulane University Medical School, New Orleans, LA, U.S.A. UDP-D-[6-^{3}H]galactose (10.1 Ci/mmol) was obtained from Amersham Corp. UDP-[6-^{3}H]*N*-

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acetyl-D-glucosamine (6.6 Ci/mmol) was purchased from New England Nuclear Corp. Pronase CB was from Calbiochem–Behring Corp; UDP-galactose, UDP-*N*-acetylglucosamine and Sephadex G-50 (Pharmacia) from Sigma Chemical Co.; and Bio-Gel P-2 from Bio-Rad Laboratories.

Cell culture

K-562 cells were obtained from Professor C. Lozzio and Professor B. Lozzio (University of Tennessee, Knoxville, TN, U.S.A.) as passage no. 211, and cultured in RPMI-1640 (Gibco) supplemented with 10% (v/v) foetal-calf serum (Gibco). All experiments were performed on cells below passage no. 260. The cultures were examined for mycoplasma by assaying for a prokaryotic DNA polymerase activity (Livingston *et al.*, 1975) and were found to be free of such contaminating organisms.

Preparation of membranes

Membrane preparations were performed at 4°C. A portion (10^9) of K-562 cells were harvested at a density of 2×10^6 cells/ml, pelleted by centrifugation (2300 *g*, 5 min) washed in sucrose buffer [0.25 M-sucrose/5 mM-Tris (pH 7.4)/0.2 mM-$MgCl_2$], re-pelleted and suspended in 20 ml of the same buffer. The cells were disrupted in a Parr nitrogen cavitation bomb after equilibration at 5.175 MPa (750 lbf/in^2) for 15 min. After disruption, the debris was removed by centrifugation at 2300 *g* for 5 min and the supernatant fraction was centrifuged at 100000 *g* for 1 h. The resulting membrane pellet was resuspended in 1 ml of Tris-buffered saline (150 mM-NaCl/20 mM-Tris, pH 7.4) and again centrifuged for 1 h. The membranes were then resuspended in 1 ml of Tris-buffered saline at a concentration of 10 mg/ml.

Standard enzyme assay and extraction

Typically the incubation mixture contained the following, in a total volume of 0.135 ml: 1.52 nmol of UDP-[^{3}H]*N*-acetylglucosamine and 5 nmol of UDP-galactose or 1 nmol of UDP-[^{3}H]galactose and 5 nmol of UDP-*N*-acetylglucosamine, 312 nmol of $MnCl_2$, 2.5 μmol of Tris/HCl, pH 7, and 1 mg of membrane suspension.

The mixture was incubated at 37°C for 30 min unless otherwise noted. The reaction was terminated by addition of 4 ml of chloroform/methanol (2:1, v/v). Ovalbumin (50 μl of a 200 mg/ml solution in 0.1 M-Tris, pH 8.0) was added as carrier protein. The mixture was vortex-mixed or sonicated and centrifuged at 3400 *g*, after which the supernatant fraction was removed. The pellet was resuspended in 4 ml of chloroform/methanol (1:2) and again centrifuged. The resulting protein-containing pellet was washed once in 5 ml of 4 mM-$MgCl_2$ (Turco *et al.*, 1980).

Preparation of glycopeptides and analysis of oligosaccharides

The protein fraction was digested exhaustively with Pronase (Turco *et al.*, 1977) to generate glycopeptides, and the glycopeptides were desalted on a column (1.5 cm × 5.0 cm) of Sephadex G-25, water being used as eluent. The desalted glycopeptides were dried and resuspended in 1 ml of 0.05 M-NaOH and 1 M-$NaBH_4$. The solution was incubated at 45°C for 16 h, neutralized with acetic acid, applied to a column (1.5 cm × 43 cm) of Sephadex G-50 and 2 ml fractions were assayed for radioactivity by liquid-scintillation spectrometry

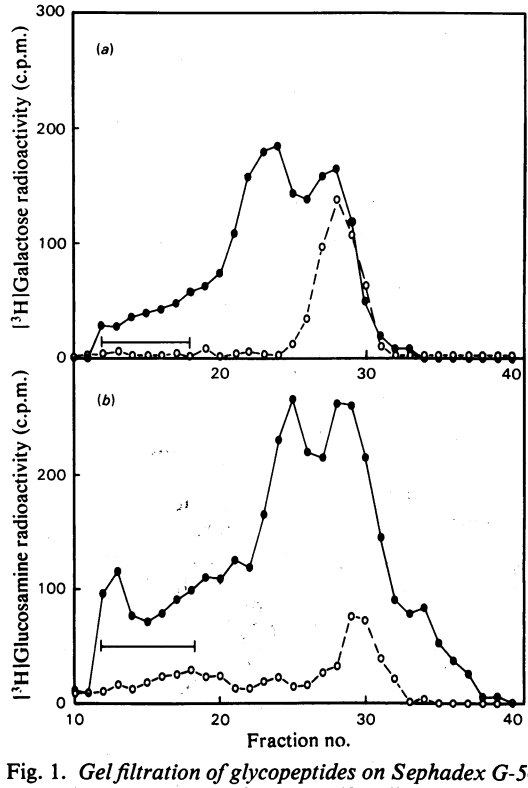


Fig. 1. *Gel filtration of glycopeptides on Sephadex G-50*
Membranes prepared from K-562 cells were incubated with either UDP-[^{3}H]galactose (10 μCi) or UDP-[^{3}H]*N*-acetylglucosamine (10 μCi) for 30 min. The mixtures were then extracted with organic solvents, the precipitate digested with Pronase, treated with alkaline $NaBH_4$, neutralized and chromatographed on a column (1.5 cm × 43 cm) of Sephadex G-50; 2 ml fractions were collected and measured for their radioactivity. Fractions 12–18 were pooled and digested with endo-β-galactosidase. (*a*) [^{3}H]Galactose-labelled glycopeptides; (*b*) [^{3}H]*N*-acetylglucosamine-labelled glycopeptides; ●, before digestion with endo-β-galactosidase; ○, pooled fractions 12–18 after digestion with endo-β-galactosidase.

(Robbins *et al.*, 1977). The high-molecular-weight glycopeptides were pooled, repeatedly dried to remove residual acetic acid, then resuspended in 0.1 ml of 50mM-sodium acetate, pH 5.8. Endo-β-galactosidase [8.33 munits (1 unit = 1 μg of reducing sugar released/h)] was added, with 10 μl of toluene to retard bacterial growth. The mixture was incubated at 37°C overnight. After digestion with endo-β-galactosidase, the glycopeptides were applied to a Sephadex G-50 column, and the fractions were assayed for radioactivity. The digestion products were then pooled and applied to a Bio-Gel P-2 column (1.0 cm × 71.0 cm), 200mM-acetic acid being used as eluent. Individual peak fractions were pooled, dried and resuspended in water.

Paper chromatography of endo-β-galactosidase-digestion products

Samples were chromatographed on Whatman no. 3 paper in a descending mode for 24–36 h using butan-1-ol/pyridine/water (6:4:3, by vol.) as a solvent system. Stachyose, raffinose, lactose, galactose and *N*-acetylglucosamine were used as standards and were detected with silver nitrate and ethanolic NaOH (Anet & Reynolds, 1954). Sections (1 cm) were cut from the chromatogram, soaked in 0.5 ml of 1% sodium dodecyl sulphate and assayed for radioactivity.

Enzymic digestions with β-galactosidase (0.92 unit) and β-*N*-acetylhexosaminidase (1.1 units) were performed in 50 μl of 50mM-sodium acetate, pH 5.8, containing 10 μl of toluene and incubated overnight at 37°C.

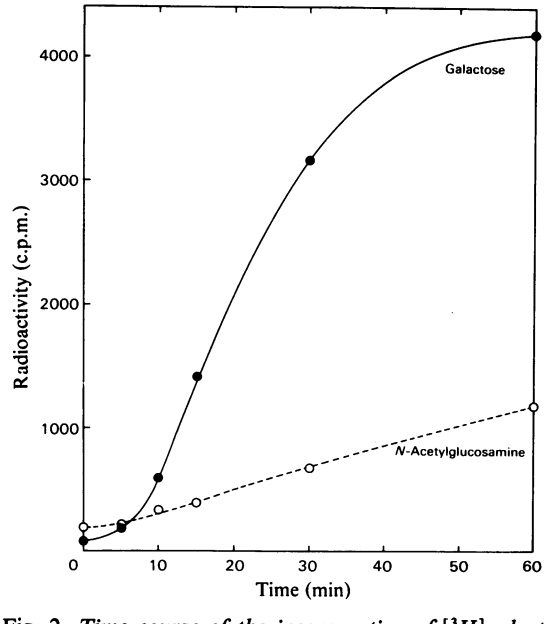


Fig. 2. *Time course of the incorporation of [³H]galactose and [³H]N-acetylglucosamine into erythroglycan*
Membranes prepared from K-562 cells were incubated with either UDP-[³H]galactose (10 μCi) or UDP-[³H]*N*-acetylglucosamine (10 μCi). At various times, portions of the mixtures were removed, extracted and treated with hydrazine (Rush *et al.*, 1981). The samples were dried, resuspended in 0.2M-acetic acid and chromatographed on Sephadex G-50. The radioactivity present in fractions 12–18 (Fig. 1) for each time point was plotted as shown.

Results

Membranes from K-562 cells were incubated in the presence of either UDP-[³H]*N*-acetylglucosamine or UDP-[³H]galactose for 30 min. Radioactive glycopeptides were prepared by extracting the membranes with organic solvents and digesting the insoluble residue with Pronase. The glycopeptides were fractionated on a column of Sephadex G-50 as shown in Fig. 1. The profiles of radiolabelled material revealed the presence of high-molecular-weight glycopeptides (fractions 12–18), comprising approx. 16% of the [³H]galactose and

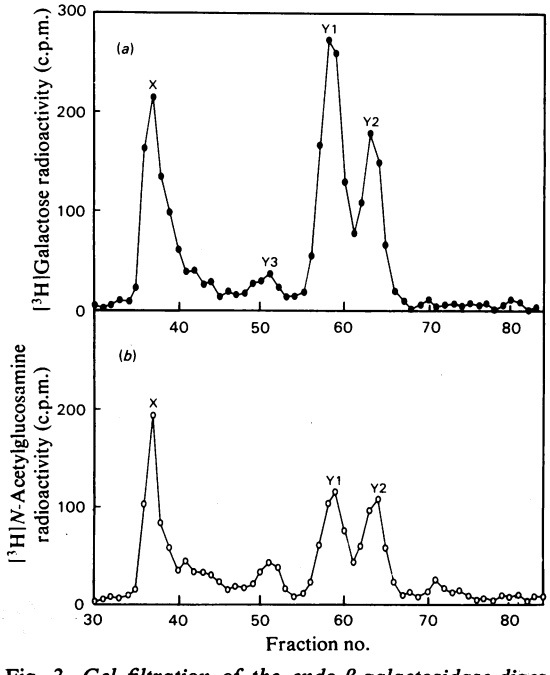


Fig. 3. *Gel filtration of the endo-β-galactosidase-digestion products on Bio-Gel P-2*
Fractions 19–32 were pooled from the Sephadex G-50 column (Fig. 1) and applied to a Bio-Gel P-2 column (1 cm × 71 cm); 0.5 ml fractions were collected. (*a*) [³H]Galactose products; (*b*) [³H]*N*-acetylglucosamine products.

19% of the [^{3}H]glucosamine glycopeptide label. These glycopeptides have an apparent mol.wt. of 7000–11000 on the basis of calibration of the column with fetuin glycopeptides (mol.wt. 3000) and a void-volume marker (bovine serum albumin). To determine whether this material was structurally related to erythroglycan (Jarnefelt *et al.*, 1978; Turco *et al.*, 1980), the glycopeptides were pooled, subjected to endo-β-galactosidase digestion and re-applied to the gel-filtration column. As shown in Fig. 1, both the [^{3}H]galactose- and [^{3}H]glucosamine-labelled glycopeptides were extensively degraded to smaller products, consistent with our previous reports (Turco *et al.*, 1980; Rush *et al.*, 1981) indicating that erythroglycan of metabolically labelled K-562 cells is readily susceptible to endo-β-galactosidase, and has thus a linear keratan-like structure.

A time course for the glycosylation *in vitro* of erythroglycan with both UDP-[^{3}H]galactose and UDP-[^{3}H]*N*-acetylglucosamine is shown in Fig. 2. After a lag time of approx. 5 min, the incorporation of both labels into erythroglycan increased for 30 min, which was followed by a gradual levelling off.

The products of endo-β-galactosidase digestion were further resolved by gel filtration on Bio-Gel P-2, as shown in Fig. 3. With either [^{3}H]galactose- or [^{3}H]*N*-acetylglucosamine-labelled material, four major products were distinguished and were named according to previous designations (Turco *et al.*, 1980). The identities of components Y1 and Y2 were determined by sequential glycosylhydrolase degradation, followed by paper-chromatographic analysis of products. As shown in Fig. 4, component Y2, labelled with either [^{3}H]galactose or [^{3}H]*N*-acetylglucosamine, migrated on paper slightly faster than the standard disaccharide lactose, with $R_{lactose} = 1.08$ (Figs. 4*a* and 4*c*). Digestion of the [^{3}H]galactose-labelled species with β-*N*-acetylhexosaminidase converted all of the label into a product that co-migrated with galactose (Fig. 4*b*), whereas digestion of the [^{3}H]*N*-acetylglucosamine-labelled material with β-*N*-acetylhexosaminidase liberated a radioactive product that co-migrated with *N*-acetylglucosamine (Fig. 4*d*). Since endo-β-galactosidase releases products with galactose at the reducing terminus, component Y2 is assigned the structure βGlcNAc-βGal.

As Fig. 5 shows, component Y1, labelled with either [^{3}H]*N*-acetylglucosamine or [^{3}H]galactose migrated on paper slightly slower than the standard trisaccharide raffinose, but faster than the tetra-

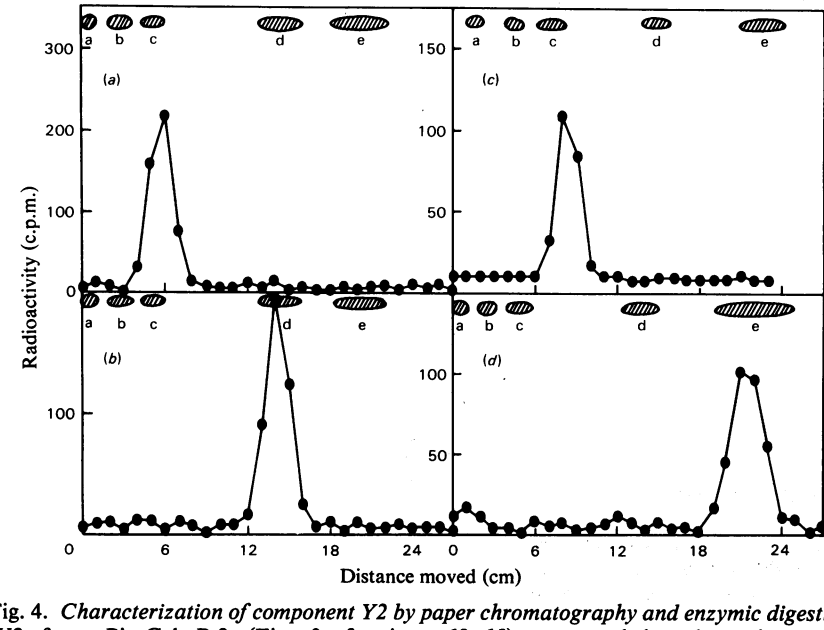


Fig. 4. *Characterization of component Y2 by paper chromatography and enzymic digestion*

Component Y2 from Bio-Gel P-2 (Fig. 3, fractions 62–65) was pooled and portions subjected to β-*N*-acetylhexosaminidase digestion. Controls and the digested material were characterized by descending paper chromatography in butan-1-ol/pyridine/water (6:4:3, by vol.). (*a*) Untreated [^{3}H]galactose component Y2; (*b*) β-*N*-acetylhexosaminidase-treated [^{3}H]galactose component Y2; (*c*) untreated [^{3}H]*N*-acetylglucosamine component Y2; (*d*) β-*N*-acetylhexosaminidase-treated [^{3}H]*N*-acetylglucosamine component Y2. Sections (1 cm) were cut out and measured for radioactivity (Robbins *et al.*, 1977). Standards: a, stachyose: b, raffinose: c, lactose: d, galactose: e, *N*-acetylglucosamine.

saccharide stachyose, with $R_{lactose} = 0.40$ (Figs. 5*a* and 5*e*). Treatment of the [^{3}H]*N*-acetylglucosamine-labelled species with β-galactosidase converted essentially all of the radioactivity to a product with $R_{lactose} = 1.10$, possessing the same mobility as component Y2 (Fig. 5*b*). Digestion of [^{3}H]-galactose-labelled component Y1 with this enzyme yielded two products, one that co-migrated with galactose and another that migrated with $R_{lactose} = 1.10$ (Fig. 5*f*). By contrast, both [^{3}H]-*N*-acetylglucosamine- or [^{3}H]galactose-labelled component Y1 were resistant to β-*N*-acetylhexosaminidase (Figs. 5*c* and 5*g*). However, incubation of [^{3}H]*N*-acetylglucosamine-labelled component Y1 with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase resulted in a labelled product migrating with *N*-acetylglucosamine. A similar digestion of the [^{3}H]galactose-labelled component Y1 liberated all of the label into a product that co-migrated with galactose. Therefore component Y1 was assigned the structure βGal-βGlcNAc-βGal.

The structural identities of both components X

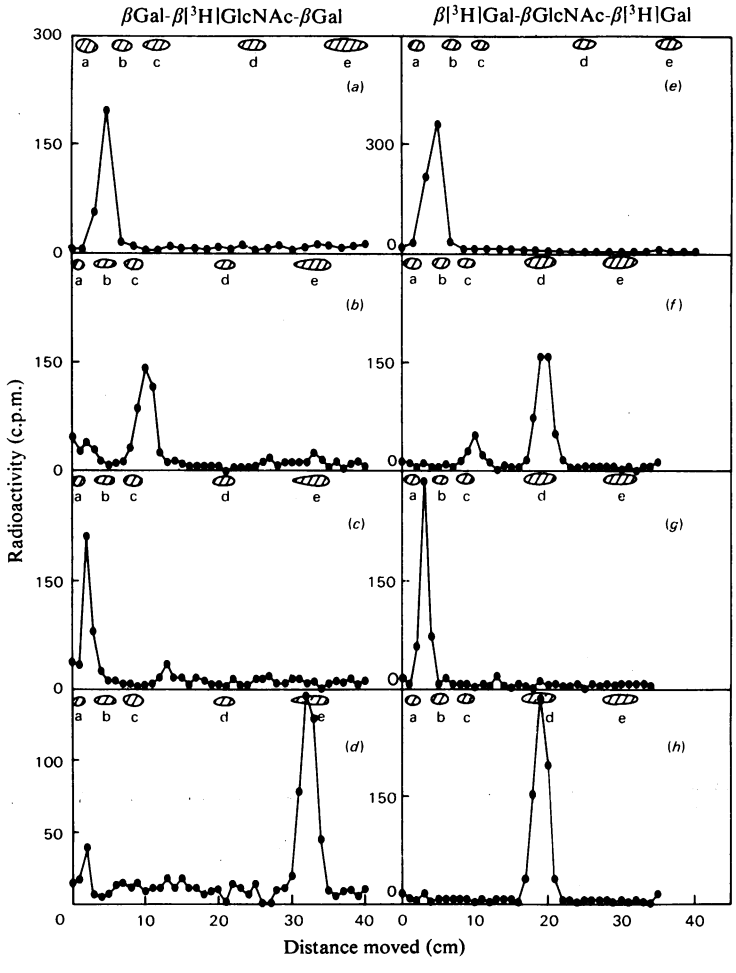


Fig. 5. *Characterization of component Y1 by paper chromatography and enzymic digestion*

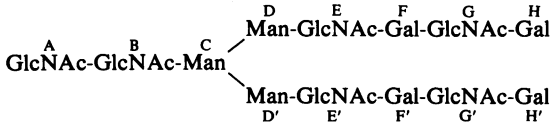
Component Y1 isolated from Bio-Gel P-2 (Fig. 3, fractions 56–60) were pooled, and portions were subjected to enzymic digestion followed by analysis by paper chromatography in butan-1-ol/pyridine/water (6:4:3, by vol.). (*a*) Untreated [^{3}H]*N*-acetylglucosamine component Y1; (*b*) β-galactosidase-treated [^{3}H]*N*-acetylglucosamine component Y1; (*c*) β-*N*-acetylhexosaminidase-treated [^{3}H]*N*-acetylglucosamine component Y1; (*d*) β-galactosidase- and β-*N*-acetylhexosaminidase-treated [^{3}H]*N*-acetylglucosamine component Y1; (*e*) untreated [^{3}H]galactose component Y1; (*f*) β-galactosidase-treated [^{3}H]galactose component Y1; (*g*) β-*N*-acetylhexosaminidase-treated [^{3}H]galactose component Y1; (*h*) β-galactosidase- and β-*N*-acetylhexosaminidase-treated [^{3}H]galactose component Y1. Sections (1 cm) were cut out of the chromatogram and assayed for radioactivity. Standards were the same as in Fig. 4.

and Y3, labelled with either precursor (Fig. 3), are as yet unknown. Component X is believed to be the protein-linkage region of the carbohydrate chain.

Discussion

The present paper contains the first documented information concerning the enzymic synthesis *in vitro* of erythroglycan. We have used a membrane preparation from K-562 cells as a source of glycosyltransferases in the transfer of [^{3}H]galactose and [^{3}H]*N*-acetylglucosamine from their UDP derivatives to endogenous erythroglycan acceptors previously described (Turco *et al.*, 1980; Rush *et al.*, 1981). The high-molecular-weight products of the transferase-catalysed reactions were partially characterized by degradation with endo-β-galactosidase, yielding: (1) the disaccharide βGlcNAc-βGal; (2) the trisaccharide βGal-βGlcNAc-βGal; (3) an unidentified oligosaccharide; and (4) a putative protein-linkage region. The latter retained some [^{3}H]galactose and [^{3}H]*N*-acetylglucosamine label, which may be the result of branched galactose residues or may be due to a sugar sequence region insensitive to endo-β-galactosidase.

It is promising that a crude microsomal membrane fraction has retained the activity for both transferases necessary for elongation of the polylactosamine chain. The fairly strict specificity of glycosyltransferases makes it clear that (if this region of the saccharide is similar to other tunicamycin-sensitive mannose-containing saccharides as shown below) the enzyme which attaches *N*-acetyl glucosamine residue G (below) to the first galactose residue (F):



must be a different enzyme from the ones that transfer *N*-acetylglucosamine residue E to the core mannose. The next galactose residue (H) may also be transferred by a unique enzyme, but this is less certain if the F–E linkage (above) is the same as the H–G linkage. The surprising information, that only one or a few proteins carry the erythroglycan structure (Rush *et al.*, 1981), whereas others carry only the more usual fetuin- or transferrin-type molecules (Kornfeld & Kornfeld, 1976), predicts that a separate signal must be encoded in the protein sequence for the action of the transferase activities described here. There must be separate, but parallel, pathways present that have strict specificity either to terminate the first lactosamine residue with sialic acid, or to elongate to erythroglycan-like structures. If a protein is found that contains both complex and erythroglycan types at defined specific locations on the same polypeptide chain, the information could be encoded in a local short primary sequence. However, if a polypeptide chain can only express either the complex or the polylactosamine-type chains, then the control for selection of the glycosylation pathway could be early in the protein sequence, where the pathways for processing must diverge.

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References

Anet, E. F. L. J. & Reynolds, T. M. (1954) *Nature (London)* **174**, 930
Drickamer, L. K. (1978) *J. Biol. Chem.* **253**, 7242–7248
Fukuda, M. N., Fukuda, M. & Hakomori, S.-I. (1979) *J. Biol. Chem.* **254**, 3700–3703
Gahmberg, C. G., Myllyla, G., Leikola, J., Pirkola, A. & Nordling, S. (1976) *J. Biol. Chem.* **251**, 6108–6116
Jarnefelt, J., Rush, J., Li, Y. T. & Laine, R. A. (1978) *J. Biol. Chem.* **253**, 8006–8009
Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* **45**, 217–237
Krusius, T., Finne, J. & Rauvala, H. (1978) *Eur. J. Biochem.* **92**, 289–300
Li, E., Gibson, R. & Kornfeld, S. (1980) *Arch. Biochem. Biophys.* **199**, 393–399
Livingston, D. M., Hinkel, D. C. & Richardson, C. C. (1975) *J. Biol. Chem.* **250**, 461–469
Parodi, A. J. & Leloir, L. F. (1979) *Biochim. Biophys. Acta* **559**, 1–37
Robbins, P. W., Krag, S. S. & Liu, T. (1977) *J. Biol. Chem.* **252**, 1780–1785
Rush, J. S., Turco, S. J. & Laine, R. A. (1981) *Biochem. J.* **193**, 361–365
Tanner, M. J. A. & Boxer, D. H. (1972) *Biochem. J.* **129**, 333–347
Turco, S. J., Stetson, B. & Robbins, P. W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4411–4414
Turco, S. J., Rush, J. S. & Laine, R. A. (1980) *J. Biol. Chem.* **255**, 3266–3269
Winzler, R. J. (1969) in *Red Cell Membrane Structure and Function* (Jamieson, G. A. & Greenwalt, T. J., eds.), pp. 157–161, J. B. Lippincott, Philadelphia