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Erythroglycan biosynthesis in K-562 cells

Inhibition of synthesis by tunicamycin and lack of attachment to the G-protein of vesicular-stomatitis virus

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K-562 cells, which express foetal erythroglycan, are shown to synthesize the lipid-linked oligosaccharide intermediates commonly found in tissues and cultured fibroblasts. The addition of tunicamycin, which blocks the formation of these intermediates and thus of asparagine-linked oligosaccharides, inhibits the synthesis of erythroglycan (M_r 7000–11000). Vesicular-stomatitis-virus infection of K-562 cells results in the glycosylation of the G-protein with the transferrin-type oligosaccharide (M_r 3000), but not with the larger erythroglycan. These results suggest that, in K-562 cells, the early stages of erythroglycan biosynthesis are the same as those of the transferrin-type oligosaccharides. However, maturation of the oligosaccharide is influenced by protein structure such that erythroglycan is only expressed on specific glycoproteins.

K-562 cells, originating from the pleural effusion of a chronic-myelogenous-leukaemia patient, have been shown to express a long-chain *N*-linked oligosaccharide (M_r 7000–11000; 'erythroglycan') on specific cell-surface glycoproteins (Turco *et al.*, 1980). The distinct feature of erythroglycan is the presence of keratan-like repeating Galβ1→4GlcNAcβ1→3 sequences attached to the polypeptide via a mannose-containing core (Järnefelt *et al.*, 1978). This cell line also synthesizes the common bi- and tri-antennary types of *N*-linked 'complex' glycopeptides such as those that can be isolated from bovine transferrin (M_r 3000) and that differ from erythroglycan by the absence of repeating *N*-acetyl-lactosamine units. The common complex chains have already been shown to be synthesized via lipid-linked oligosaccharide intermediates (Parodi & Leloir, 1979), and the present study, with the use of the antibiotic tunicamycin, provides evidence that the larger complex chains are synthesized in a similar manner.

In addition, we have examined vesicular-stomatitis-virus-infected K-562 cells in order to evaluate the selectivity of *N*-glycosylation of the viral glycoprotein in a cell line known to produce several kinds of *N*-linked saccharides. Vesicular-stomatitis virus is a lipid-enveloped RNA virus containing one glycoprotein (G-protein) in its membrane. It is an appropriate model system to address this aspect, since normal host protein, as well as RNA synthesis, is inhibited during viral infection (Etchison *et al.*, 1977). The G-protein is reported to be glycosylated with two transferrin-type oligosaccharides by host enzymes in fibroblastic cell lines such as BHK (baby-hamster kidney) (Reading *et al.*, 1978). Thus we decided to determine whether the G-protein could be glycosylated with the erythroglycan chain in K-562 cells.

In the present paper we show that the G-protein is glycosylated in K-562 cells only with oligosaccharides of approx. $M_r \simeq 3000$ and not with the longer-chain erythroglycan. The results indicate that there are at least two pathways of synthesis via lipid-linked intermediates of *N*-linked glycopeptides of the complex type in K-562 cells. The G-protein of vesicular-stomatitis virus appears to be selectively glycosylated by the transferrin-type pathway, leading to typical bi- and tri-antennary oligosaccharide structures (Kornfeld & Kornfeld, 1976).

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Materials and methods

Materials

Endo-β-galactosidase from *Escherichia freundii* was generously given by Professor Y.-T. Li (Tulane University, New Orleans, LA, U.S.A.). Other materials were as follows: [6-^{3}H]glucosamine (18.8 Ci/mmol), [2-^{3}H]mannose (18.4 Ci/mmol) and [^{3}H]leucine (51.6 Ci/mmol) from New England Nuclear Corp.; Bio-Gel P-4 from Bio-Rad Laboratories; Sephadex G-50 from Sigma. Tunicamycin

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was obtained from Dr. Robert Hamill of Eli Lilly Co.

Cell culture

K-562 cells were obtained from Professor C. B. Lozzio (University of Tennessee, Knoxville, TN, U.S.A.) as passage no. 211. The cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) foetal-calf serum (Gibco). Cell cultures were determined to be free of mycoplasma as described by Livingston *et al.* (1975). All experiments were performed on cells below passage no. 240.

Metabolic labelling and extraction

Exponentially growing cells (10^6 cells/ml) were centrifuged (1000 *g*) for 5 min to remove the medium and washed with 10 ml of phosphate-buffered saline (0.8% NaCl/0.02% KCl/0.115% Na_2HPO_4/0.2% KH_2PO_4/0.01% $CaCl_2$/0.01% $MgCl_2$). The cells (10^7) were incubated in 1 ml of Dulbecco's modified Eagle's medium containing 2% of the normal concentration of glucose (20 μg/ml) and [^{3}H]glucosamine (20 μCi/ml) or [^{3}H]mannose (20 μCi/ml) and supplemented with 10% dialysed foetal-calf serum and glutathione (1 μg/ml). After 18 h of incubation at 37°C, the cells were separated from the medium by centrifugation, washed with 10 ml of phosphate-buffered saline and the labelled proteins extracted with chloroform/methanol/water (3:2:1, by volume). The insoluble residue obtained by extraction of the [^{3}H]mannose-labelled cells was washed with water and extracted with chloroform/methanol/water (10:10:3, by volume), which solubilized the oligosaccharide–lipid fraction (Turco *et al.*, 1977).

Preparation of protein-derived oligosaccharides

The oligosaccharides were released from the lipid-extracted pellet by hydrazinolysis with freshly distilled anhydrous hydrazine at 100°C for 20 h (Bayard & Roux, 1975). The hydrazine was removed by rotary evaporation under reduced pressure and by drying, under a stream of N_2, with toluene three to five times. The amino sugars in the oligosaccharides were re-*N*-acetylated by treatment with pyridine/acetic anhydride (1:1, v/v) for 1 h at 100°C, followed by de-*O*-acetylation with 0.1 M-KOH in toluene/methanol (1:3, v/v) at room temperature for 4 h. The oligosaccharides were then reduced with 1 M-NH_3/0.1 M-$NaBH_4$, neutralized with acetic acid and chromatographed on Sephadex G-50.

Vesicular-stomatitis-virus infection of cells

Exponentially growing K-562 (2×10^5–20×10^5 cells/ml) or BHK cells (2×10^5–20×10^5 cells/60 mm-diam. plate) were infected with vesicular-stomatitis virus (multiplicity of infection = 5–10). At 5 h after infection the medium was removed and the cells were incubated for 60 min in Dulbecco's modified Eagle's medium supplemented with dialysed 10% foetal-calf serum and the appropriate radioisotopically labelled amino acid or sugar. Then the medium was removed and the cells were washed twice with phosphate-buffered saline. The cells were precipitated with 5% trichloroacetic acid and washed with ice-cold acetone.

The radiolabelled proteins were dissolved and separated by polyacrylamide-gel electrophoresis as described by Laemmli (1970). The separating gel was 10% acrylamide and the stacking gel was 3.6% acrylamide. Electrophoresis was carried out for 5 h at 25 mA. After electrophoresis, the gels were subjected to fluorography as described by Bonner & Laskey (1974), Kodak XR-5 film being used.

Results

The pathway of biosynthesis of erythroglycan is unknown; however, preliminary information concerning its expression in K-562 cells was obtained by examining the distribution of lipid-linked oligo-

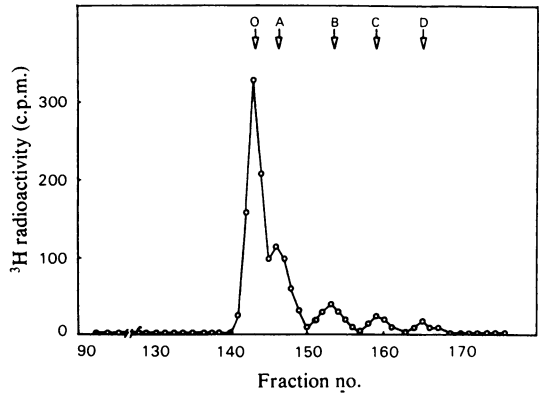

Fig. 1. *Bio-Gel P-4 chromatogram of K-562-cell-derived [^{3}H]mannose-labelled oligosaccharides released from lipid by mild acid hydrolysis*

The [^{3}H-*Man*]oligosaccharide–lipid fraction, obtained as described in the Materials and methods section, was subjected to mild acid hydrolysis (Turco *et al.*, 1977), treated with endo-β-*N*-acetylglucosaminidase H (Robbins *et al.*, 1977), mixed with [^{14}C-*Man*]oligosaccharide standards (not plotted) and applied to a column (1 cm × 240 cm) of Bio-Gel P-4 (400 mesh); 0.5 ml fractions were collected. Letters above the peaks refer to the elution positions of standards (Liu *et al.*, 1979): O, $Glc_3Man_9GlcNAc$; A, $Glc_2Man_9GlcNAc$; B, $Glc_1Man_9GlcNAc$; C, $Man_9GlcNAc$; D, $Man_8GlcNAc$.

saccharide intermediates synthesized in these cells and by determining the effect of tunicamycin on metabolic carbohydrate labelling of high-molecular-weight oligosaccharides. As shown in Fig. 1, the profile of radioactivity from [^{3}H]mannose labelling *in vivo* of lipid-linked oligosaccharides is similar to that reported in numerous cell systems (Robbins *et al.*, 1977). Thus the largest labelled lipid-derived oligosaccharide is believed to be $Glc_3Man_9GlcNAc_2$, with lesser amounts of smaller lipid-derived oligosaccharides thought to be precursors to $Glc_3Man_9GlcNAc_2$–lipid intermediate. With variable labelling times, oligosaccharides of unusual size were not observed.

Tunicamycin inhibits the glycosylation of glycoproteins with *N*-linked oligosaccharides by blocking the formation of dolichyl pyrophosphate *N*-acetylglucosamine and consequently all *N*-acetylglucosamine–lipid intermediates (Hickman *et al.*, 1977). As previously reported (Turco *et al.*, 1980), metabolic labelling of K-562 cells with [^{3}H]-mannose results in the incorporation of label into glycoproteins whose glycopeptides can be separated into two major size classes by gel filtration on Sephadex G-50; 'erythroglycan' (M_r 10000), and bi- and tri-antennary structures of the transferrin-type (M_r 3000). As shown in Fig. 2, tunicamycin inhibits the incorporation of [^{3}H]mannose into both of these size classes to comparable extents. At a tunicamycin concentration of 4μg/ml, specific radioactivity (c.p.m./μg of cell protein) found in erythroglycan decreased by 75% and in transferrin-type structures by 82%. At this concentration of tunicamycin, total cellular protein was only 14% less than that present in control cultures, indicating that the primary effect of the antibiotic is on glycoprotein glycosylation. Increasing the tunicamycin concentration to 10μg/ml had no further significant effect on synthesis of either erythroglycan or the 3000-M_r saccharides. The incorporation of [^{3}H]glucosamine into erythroglycan was decreased by 66% at 4μg of tunicamycin/ml; no further decrease in synthesis was

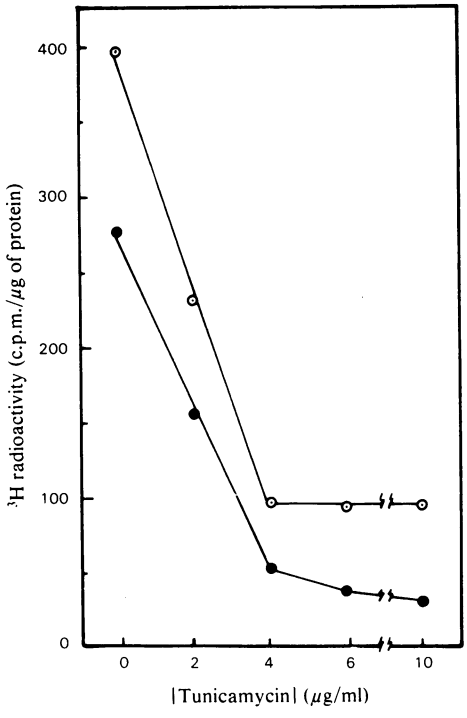

Fig. 2. *Inhibition of synthesis of protein-derived oligosaccharides by tunicamycin*

K-562 cells (2×10^6 cells) were preincubated with tunicamycin for 2h and labelled with [^{3}H]glucosamine (20μCi) for 16h in the presence of tunicamycin. After labelling, protein-derived [^{3}H]-mannose-labelled oligosaccharides were prepared as described in the Materials and methods section and subsequently chromatographed on Sephadex G-50 (Turco *et al.*, 1980). Protein was determined from duplicate plates of cells. The radioactivity (c.p.m./μg of protein) in oligosaccharides of M_r 7000–11000 (⊙) and 3000 (●) is shown.

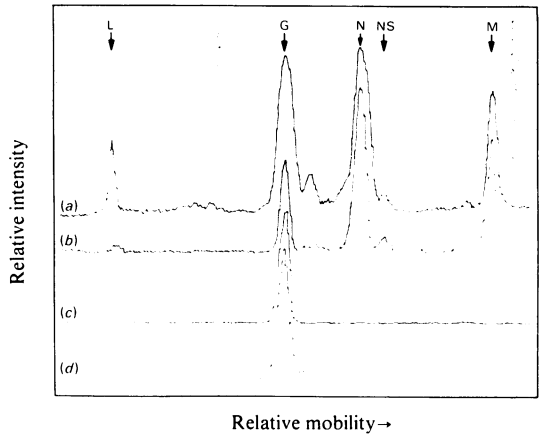

Fig. 3. *Gel profiles of radiolabelled proteins of vesicular-stomatitis-virus-infected K-562 and BHK cells*

Radiolabelled proteins were obtained as described in the Materials and methods section. Shown are soft-laser-densitometer tracings of a fluorogram (7 days exposure). L, G, N, NS and M indicate the proteins of vesicular-stomatitis virus, as described by Wagner *et al.* (1972). (*a*) [^{3}H]Leucine-labelled vesicular-stomatitis-virus-infected K-562 cells; (*b*) [^{3}H]leucine-labelled vesicular-stomatitis-virus-infected BHK cells; (*c*) [^{3}H]mannose-labelled vesicular-stomatitis-virus-infected K-562 cells; (*d*) [^{3}H]glucosamine-labelled vesicular-stomatitis-virus-infected K-562 cells.

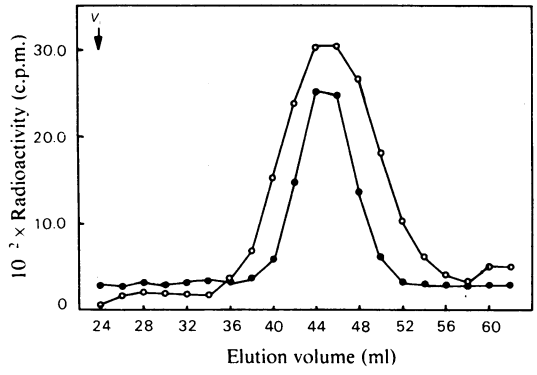

Fig. 4. *Gel filtration of G-protein glycopeptides on Sephadex G-50*

[^{3}H]Mannose- and [^{3}H]glucosamine-labelled G-protein from vesicular-stomatitis-virus-infected K-562 cells were excised from a polyacrylamide gel (Fig. 3), freed of the polyacrylamide by electrophoresis, precipitated with 5% (w/v) trichloroacetic acid, washed twice with ice-cold acetone, and the glycopeptides prepared by Pronase digestion. The Sephadex G-50 column (1.5cm × 40cm) was eluted with 0.1M-pyridine/acetic acid, pH5.0. Fractions of 2.0ml were collected and radioactivity was monitored by liquid-scintillation counting of appropriate samples. ○, [^{3}H]Mannose-labelled G-protein glycopeptides; ●, [^{3}H]glucosamine-labelled G-protein glycopeptides.

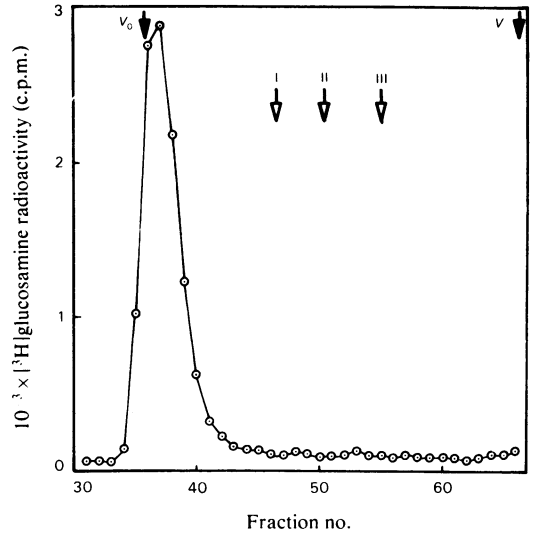

Fig. 5. *Gel filtration of endo-β-galactosidase-digested [^{3}H]glucosamine-labelled G-protein glycopeptides on Bio-Gel P-2*

[^{3}H]Glucosamine-labelled G-protein glycopeptides from vesicular-stomatitis-virus-infected K-562 cells were treated with 200munits of endo-β-galactosidase in 0.05M-sodium acetate, pH5.8, for 18h. The sample was then applied to a column (1.5cm × 100cm) of Bio-Gel P-2 and eluted with 15% (v/v) acetic acid. Fractions of 1.5ml were collected. I, II and III indicate the elution volumes of authentic tetrasaccharide (αAcNeu1→3,6βGal1→4βGlcNAc1→3-βGal), trisaccharide (βGal1→4βGlcNAc1→3-βGal), and disaccharide (βGlcNAc1→3βGal) isolated previously (Turco *et al.*, 1980).

seen up to 10μg/ml of tunicamycin. These results strongly suggest that the tunicamycin-sensitive biosynthetic steps leading to the common *N*-linked glycopeptides (transferrin-type) are the same as the initial stages of erythroglycan synthesis.

As discussed in the introduction, we decided to determine whether erythroglycan could be attached to the G-protein of vesicular-stomatitis-virus-infected K-562 cells, since these cells potentially could glycosylate the G-protein with the smaller transferrin-type, as is the case with BHK cells or with the long-chain versions of 'complex' units (erythroglycan). As seen in the densitometer scans of radioautograms of sodium dodecyl sulphate/polyacrylamide gels in Fig. 3, 5.5h after infection of K-562 cells (*a*) with vesicular-stomatitis virus the same proteins are radiolabelled with [^{3}H]leucine as in vesicular-stomatitis-virus-infected BHK cells (*b*). Fig. 3 further reveals that only the G-protein of vesicular-stomatitis-virus-infected K-562 cells is labelled when either [^{3}H]mannose (*c*) or [^{3}H]-glucosamine (*d*) is incubated with the cells 5.5h after infection. Fig. 4 shows the profile of the glycopeptides derived from Pronase-digested [^{3}H]glucosamine and [^{3}H]mannose-labelled G-protein from vesicular-stomatitis-virus-infected K-562 cells. All of the resultant glycopeptides are co-eluted with standard glycopeptides of M_r 3000 isolated from bovine transferrin. No glycopeptides of M_r = 7000–11000 (the size of erythroglycan) were obtained. Consistent with this result, the [^{3}H]glucosamine-labelled G-protein glycopeptides were found to be resistant to degradation by endo-β-galactosidase from *E. freundii* as shown in Fig. 5. This enzyme is specific for keratan-like molecules with a repeating *N*-acetyl-lactosamine structure of Galβ1→4GlcNAc-β1→3. No di-, tri- or tetra-saccharide products were obtained as for foetal erythroglycan (Fukuda *et al.*, 1979; Turco *et al.*, 1980), indicating the absence of the GlcNAcGal repeating unit. From these data we conclude that K-562 cells do not glycosylate the G-protein with erythroglycan *N*-acetyl-lactosamine repeating units.

Discussion

In the biosynthetic scheme for glycosylation of protein with *N*-linked saccharides [see Parodi &

Leloir (1979) for review], the initial glycosylation is achieved by the transfer of the $Glc_3Man_9GlcNAc_2$ oligosaccharide from a lipid intermediate to an asparagine residue on the protein. The pathways leading to the formation of this lipid-linked oligosaccharide can be blocked by tunicamycin (Hickman *et al.*, 1977).

In our experiments, the synthesis of both erythroglycan and transferrin-type glycopeptides is inhibitable by tunicamycin to the same extent, suggesting that both proceed via similar pathways using lipid-linked intermediates. The observation that specific glycoproteins of K-562 cells and erythrocyte membranes are not randomly glycosylated with all available *N*-linked chains, but specifically with erythroglycan, suggests that a strictly controlled branch point in the synthetic mechanisms must exist. In the accepted scheme for the synthesis of the transferrin-type oligosaccharides, glucose and mannose residues are removed sequentially from the non-reducing ends of the protein-bound $Glc_3Man_9GlcNAc_2$ oligosaccharide, and the mannose core is then elaborated with two to four disaccharides consisting of Galβ1→4GlcNAc. Each of these disaccharides is then normally terminated by an *N*-acetylneuraminic acid residue (Schachter *et al.*, 1970) before leaving the Golgi apparatus. In the case of erythroglycan, the repeating chain of Galβ1→4GlcNAcβ1→3 is presumably added to the mannose core region in a series of additions to produce the higher oligomers found in erythrocyte membranes (Järnefelt *et al.*, 1978; Krusius *et al.*, 1978). Information encoded in the nascent protein must signal whether the processed oligosaccharide chain will be elongated to produce erythroglycan or terminated by *N*-acetylneuraminic acid. The glycosyltransferase systems of the endoplasmic reticulum must somehow be sensitive to this information in order to accomplish the observed selective glycosylation.

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