Glycoprotein biosynthesis in quiescent and stimulated thymocytes and a T-cell lymphoma

The synthesis of high-mannose oligosaccharides

C. Anthony RUPAR and Geoffrey M. W. COOK

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD, U.K.

(Received 7 September 1981/Accepted 9 October 1981)

Quiescent thymocytes, mitogen-stimulated thymocytes and acute-leukaemic lymphoblasts provide a model for the study of protein glycosylation in quiescent cells, mitotically active non-malignant and malignant cells respectively. The biosynthesis of both complex and high-mannose-type oligosaccharides was monitored by metabolic labelling with [6-³H]fucose and [2-³H]mannose. Bio-Gel P6 elution profiles of [6-³H]fucose-labelled glycopeptides showed that quiescent thymocytes and stimulated thymocytes synthesized qualitatively and quantitatively similar glycopeptides; however, higher-molecular-weight glycopeptides were synthesized by the acute-leukaemic lymphoblasts. The amount of [2-³H]mannose incorporated into glycopeptide by quiescent thymocytes was less than 10% of that incorporated by stimulated thymocytes. The Bio-Gel P6 elution profile of [2-³H]mannose-labelled glycopeptides from acute-leukaemic lymphoblasts was qualitatively similar to that of stimulated thymocytes, with about 40% of the radioactivity incorporated into one glycopeptide peak. This glycopeptide was characterized by Bio-Gel P6 and concanavalin-A affinity chromatography, radioactive-sugar analysis, sensitivity to α -mannosidase and endoglycosidase H and resistance to β -glucosaminidase as containing a high-mannose oligosaccharide, possibly of Man₇₋₈GlcNAc₂ structure. Pulse/chase experiments indicated that this high-mannose oligosaccharide was an end product and not a biosynthetic intermediate. It is concluded that higher-molecular-weight fucose-labelled glycopeptides are characteristic of the malignant cell type, and the synthesis of high-mannose oligosaccharide, Man₇₋₈GlcNAc₂, in stimulated thymocytes and acute-leukaemic lymphoblasts is associated with mitotically active cells.

Cell-surface carbohydrates are considered to perform an important role in regulating aspects of cell social behaviour. Alterations in these structures which may occur in normal and malignant cells are of particular interest in efforts to assign specific functions to cell-surface carbohydrates. Changes in glycopeptide structure relevant to neoplastic growth have been investigated most often *in vitro* with transformed cells, and the consistent observation has been an increase in the size of fucose-containing cell-surface glycopeptides, possibly owing to increased sialic acid content [reviewed by Warren *et al.* (1978) and Atkinson & Hakimi (1980)]. Further experiments have indicated this phenomenon to be of a quantitative rather than qualitative nature, with alterations present in the structures of carbohydrates on a large number of cell-surface glycoproteins (Baker *et al.*, 1980; Blithe *et al.*, 1980).

Cell-surface glycopeptides of rapidly growing and density-inhibited cells (reviewed by Atkinson & Hakimi, 1980) have structural alterations which are similar to those described in transformed cells with an increase in higher-molecular-weight fucose-containing glycopeptides (Buck *et al.*, 1971; Ceccarini, 1975). A second type of alteration in the structure of cell-surface glycopeptides of rapidly growing cells is the cell-cycle-dependent appearance of high-mannose oligosaccharides (Ceccarini *et al.*, 1975; Muramatsu *et al.*, 1976). Transformed cells also have more high-mannose oligosaccharides than do control cell lines (Ceccarini & Atkinson, 1977).

Comparative studies of glycosylation patterns in systems *in vitro* are complicated by difficulties in defining a 'normal' cell and distinguishing between transformed cells and malignant cells. As discussed by Atkinson & Hakimi (1980), the use of some cell

lines as 'normal' cells and their virally transformed counterparts as models for malignant cells suffers from the fact that all cell lines may not have the limited life-span associated with a 'normal' cell.

Quiescent thymocytes and mitogen-stimulated thymocytes, in primary culture, constitute an experimental system for studying protein glycosylation in 'normal' diploid cells of finite life-span in either a quiescent or a mitotically active state. In addition, primary cultures of acute leukaemic lymphoblasts of T-cell origin in AKR mice are malignant cells which when implanted subcutaneously or intraperitoneally kill a host within 14 days. In this paper, the biosynthesis of oligosaccharides which are *N*-glycosidically linked to glycoproteins has been studied in quiescent thymocytes, mitogen-stimulated thymocytes and acute-leukaemic lymphoblasts in an attempt to differentiate between growth-dependent and malignancy-dependent alterations in the biosynthesis of cell-surface carbohydrates. Metabolic labelling with [2-³H]mannose and [6-³H]fucose has been used to monitor, respectively, the biosynthesis of high-mannose oligosaccharides, which contain mannose and *N*-acetylglucosamine, and complex oligosaccharides, which contain fucose, mannose, galactose, *N*-acetylglucosamine and sialic acid. The results indicate that high-mannose oligosaccharides are preferentially synthesized in both mitogen-stimulated thymocytes and acute-leukaemic lymphoblasts, and an increase in molecular weight of fucosylated glycopeptides is associated only with the malignant cells.

Materials and methods

Preparation of thymocytes and AKR acute leukaemic lymphoblasts

Thymocytes were isolated from 3–5-month-old 'R' strain mice, a substrain of AKR mice which is resistant to the AKR lymphoma (Cook & Jacobson, 1968). Suspensions of thymocytes were prepared by pressing the thymus through a fine stainless-steel gauze (0.5 mm mesh) and subsequent washing with RPMI 1640 medium (Flow Laboratories, Irvine, Scotland, U.K.) Viability was greater than 95% as determined by exclusion of Trypan Blue.

The acute lymphoblastic leukaemia was transmitted in AKR mice (Olac Ltd., Bicester, Oxon., U.K.) as described in detail by Cook & Jacobson (1968). Tumours were removed 12 days after subcutaneous implantation and a cell suspension was prepared by mechanical dissociation through a stainless-steel gauze (0.8 mm mesh) in RPMI 1640 medium. A population highly enriched in acute leukaemic lymphoblasts was obtained by centrifuging the cell suspension on an Isopaque/Ficoll barrier and recovering the cells collecting at the

interphase as described by Davidson & Parish (1975). The cell suspensions prepared in this manner were free of erythrocytes and macrophages and contained less than 14% B lymphocytes as assessed by immunocytoadherence (M. McClure, C. A. Rupar & G. M. W. Cook, unpublished work). Approx. 2×10^7 cells with 90% viability, as estimated by exclusion of Trypan Blue, were obtained from each 0.5 g (wet weight) tumour.

[³H]Thymidine incorporation

Microtitre-plate (Flow Laboratories) cultures were used to determine the time course of mitogenic stimulation by concanavalin A (thrice crystallized; Miles Laboratories, Slough, U.K.). Thymocytes (5×10^6 cells/ml) were maintained at 37°C in RPMI 1640 medium supplemented with penicillin (50 units/ml) streptomycin (50 µg/ml) and L-glutamine (300 mg/l). Mitogenic stimulation was assayed by adding indicated amounts of concanavalin A and [³H]thymidine (0.4 µCi; 45 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) dissolved in RPMI 1640 medium (10 µl) to cell suspensions (200 µl containing 1×10^6 cells). After 8 h in the presence of [³H]thymidine, the incorporation of radioactivity into acid-insoluble material was determined by transferring the cell suspension to a conical centrifuge tube (1.5 ml), adding bovine serum albumin (300 µl of solution containing 1 mg/ml of RPMI 1640 medium) as carrier, and precipitating immediately with cold 10% (w/v) trichloroacetic acid (500 µl). The precipitate was washed twice with cold 10% (w/v) trichloroacetic acid (500 µl) and solubilized with Soluene (0.5 ml; Packard Instrument Co., Downers Grove, IL, U.S.A.), at room temperature. Radioactivity was determined in a scintillation mixture containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.1 g/l) in toluene/2-ethoxyethanol (7:3, v/v).

Metabolic labelling of cells

Quiescent and mitogen-stimulated thymocytes were maintained in RPMI 1640 medium supplemented with penicillin (50 units/ml) and streptomycin (50 µg/ml) at cell concentrations of 5×10^6 cells/ml in a total volume of 75 ml in sealed 75 cm² tissue-culture flasks. Mitogen and either [2-³H]mannose (12 Ci/mmol) or [6-³H]fucose (22 Ci/mmol; both obtained from The Radiochemical Centre) were dissolved in RPMI 1640 before addition to the cultures. [2-³H]Mannose was supplied in an aq. 50% (v/v) ethanol solution and the ethanol was removed under a stream of nitrogen before use. Labelled sugars were added to the cultures at a final concentration of 1 µCi/ml.

The leukaemic cells were metabolically labelled, in a similar manner, while maintained at 8×10^6

cells/ml in a total volume of 2.5 ml. Radioactive substrates were added at a final concentration of $10\mu\text{Ci/ml}$.

Cells were harvested at the end of the labelling period by centrifugation (5 min, 500g) and washed twice with RPMI 1640 (10 ml).

Pulse/chase metabolic labelling of cells

Cells were metabolically labelled as described above for a 12 h pulse period, harvested (5 min, 500g) and washed twice in RPMI 1640 before transfer to fresh media without the radioactive substrate for the 12 h chase period.

Preparation of glycopeptides containing N-glycosidically linked oligosaccharides

Washed cells were suspended in RPMI 1640 (1 ml) and total lipid was extracted with chloroform/methanol as described by Bligh & Dyer (1959). The precipitate remaining after delipidation of [$2\text{-}^3\text{H}$]mannose-labelled cells was suspended in 0.1 M-Tris/HCl (pH 7.9)/1 mM- CaCl_2 containing Pronase (100 μg , B grade, Calbiochem) for 4 days at 37°C under a layer of toluene. Further additions of Pronase (100 μg) were made every 24 h.

Lipid was extracted from cells labelled with [$6\text{-}^3\text{H}$]fucose (Bligh & Dyer, 1959) and the remaining residue suspended in 0.1 M-NaOH/0.8 M- NaBH_4 (1 ml) at 37°C for 48 h to remove O-glycosidically linked oligosaccharides (Spiro & Bhoyroo, 1974). Excess NaBH_4 was destroyed by the dropwise addition of acetic acid and then bovine serum albumin (200 μl ; 1 mg/ml in 0.9% NaCl) was added as a carrier protein and total protein was precipitated by the addition of an equal volume of cold 1.0 M-HCl/2% (w/v) phosphotungstic acid. Precipitates were washed twice with cold 1.0 M-HCl/2% (w/v) phosphotungstic acid and suspended in 0.1 M-Tris/HCl (pH 7.9)/1 mM- CaCl_2 (1 ml). Digestion with Pronase was performed as described above.

Gel-filtration chromatography

After Pronase digestion, samples were kept at 100°C for 15 min, cooled and centrifuged (5 min, 500g), and the supernatant was then placed on a column (1.5 cm $\times$ 90 cm) of Bio-Gel P6 (200–400 mesh; Bio-Rad Laboratories). The column was eluted with 0.1 M-ammonium acetate, pH 7, containing 0.02% NaN_3 , at room temperature with a flow rate of 7 ml/h. Fractions (2.5 ml) were collected and 0.5 ml samples removed for radioactivity determination in a scintillation mixture containing 2,5-diphenyloxazole (5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.5 g/l) in toluene/Triton X-100 (2:1, v/v) and the remainder of each fraction was kept for subsequent analysis.

The Bio-Gel P6 column was calibrated with Blue Dextran, large oligosaccharide from fetuin and its

desialylated derivative, stachyose and mannose, with respective molecular weights of >6000, 2878, 2005, 667 and 180. The large oligosaccharide of fetuin (Gibco, Grand Island, NY, U.S.A.) was a gift from B. Bendiak of this laboratory and was prepared by the hydrazinolysis of fetuin by the procedure of Mizuochi *et al.* (1978). The desialylated oligosaccharide was prepared by hydrolysis in 0.05 M- H_2SO_4 at 80°C for 1 h. Molecular weights were assigned to the fetuin-derived oligosaccharides on the basis of the structure described by Nilsson *et al.* (1979).

A plot of K_{av} versus $\ln(\text{molecular weight})$ showed a linear relationship for the four higher-molecular-weight markers; mannose was eluted below the fractionation range of this column.

Affinity chromatography

Affinity chromatography was performed on immobilized concanavalin A by using a column (10 cm $\times$ 0.5 cm) of Glycosylex A (Miles Laboratories) prepared in 0.1 M-ammonium acetate, pH 7.0, made 1 mM with respect to CaCl_2 , MnCl_2 and MgCl_2 and containing 0.02% (w/v) NaN_3 . Samples were allowed to equilibrate on the column for 1 h at room temperature before elution. Routinely the columns were eluted with 10 ml of 0.1 M-ammonium acetate, pH 7.0, made 1 mM with respect to CaCl_2 , MnCl_2 and MgCl_2 and containing 0.02% (w/v) NaN_3 , then by a linear gradient (20 ml total volume) of 0–0.08 M- α -methyl glucoside and finally with 0.25 M- α -methyl glucoside (20 ml), both in the same buffer. Fractions (1.5 ml) were collected and counted for radioactivity in the same Triton X-100-based scintillation mixture as used for gel filtration chromatography.

Monosaccharide analysis

Radioactively labelled glycopeptides were hydrolysed in 0.5 M- H_2SO_4 for 8 h at 100°C and the radioactive sugars separated by descending paper chromatography on Whatman 3MM paper in butan-1-ol/ethanol/water (10:1:2, by vol.) solvent as described by Spiro (1966). Guide lanes of standard sugars were located at both edges of each chromatogram and detected with aniline/phthalate reagent (Partridge, 1949). Areas from sample lanes corresponding to the standards were cut out and placed in water (1 ml) overnight. Samples were counted for radioactivity after the addition of 9 ml of a Triton X-100-based scintillation cocktail.

Glycosidase treatment

Solutions of radioactive glycopeptides (0.26 ml) were treated with α -mannosidase (EC 3.2.1.24; 0.07 units; Boehringer Mannheim), β -N-acetylglucosaminidase (EC 3.2.1.30; 0.06 units, Sigma) and endoglycosidase H (EC 3.2.1.96; 0.002 unit; Miles Laboratories) in 0.1 M-ammonium acetate, pH 5.0,

containing 0.02% (w/v) NaN_3 for 20 h at 37°C. Reactions were stopped by heating at 100°C for 15 min, cooled, and centrifuged (5 min, 500 g), and the supernatant was chromatographed on the Bio-Gel P6 column. α -Mannosidase was tested for β -*N*-acetylglucosaminidase activity and β -*N*-acetylglucosaminidase was tested for α -mannosidase activity against the appropriate *p*-nitrophenyl glycoside substrates as described by Li & Li (1972). The α -mannosidase contained about 2% β -*N*-acetylglucosaminidase activity and the β -*N*-acetylglucosaminidase contained no detectable α -mannosidase activity.

Results

Mitogenic stimulation kinetics

The kinetics of mitogenic stimulation of thymocytes by the polyclonal T-cell mitogen, concanavalin A, were studied by determining [^3H]thymidine incorporation into trichloroacetic acid-insoluble material. Microtitre-plate cultures of thymocytes were established in the presence or absence of concanavalin A (1 $\mu\text{g}/\text{ml}$) and given an 8 h pulse of [^3H]thymidine before harvest at the times indicated in Fig. 1. Incorporation of [^3H]thymidine into trichloroacetic acid-insoluble material increases in concanavalin A-stimulated thymocytes, relative to quiescent thymocytes, after about 24 h exposure to concanavalin A, rising to a 38-fold difference at 48 h. This incorporation of [^3H]thymidine into acid-insoluble material, presumably DNA, indicates that the mitogen-stimulated thymocytes are entering S phase of the cell cycle in preparation for mitotic division, whereas the non-stimulated thymocytes remain in G_1 phase after 48 h in culture. Concentrations of concanavalin A from 0.2 to 6.4 $\mu\text{g}/\text{ml}$ were tested for both mitogenic activity and effects on cell viability, and 1 μg of concanavalin A/ml was found to give optimal stimulation without affecting cell viability. Concentrations of concanavalin A greater than 3 $\mu\text{g}/\text{ml}$ were cytotoxic (results not shown).

[$2\text{-}^3\text{H}$]Mannose incorporation into glycopeptides of quiescent thymocytes, concanavalin A-stimulated thymocytes and acute-leukaemic lymphocytes

Quiescent thymocytes, concanavalin A-stimulated thymocytes and leukaemic cells were each maintained in the presence of [$2\text{-}^3\text{H}$]mannose for 0–12 h and 12–24 h labelling periods. Concanavalin A was present in stimulated thymocyte experiments from 0 h. In Figs. 2(a) and 2(b) the elution profiles obtained on Bio-Gel P6 of Pronase-digested [$2\text{-}^3\text{H}$]mannose-labelled glycopeptides after a 0–12 h labelling period are shown. Equal numbers of quiescent and stimulated thymocytes were used; however, the amount of radioactivity incorporated

Fig. 1. [^3H]Thymidine incorporation into stimulated thymocytes and quiescent thymocytes

Concanavalin A-stimulated thymocytes and quiescent thymocytes were labelled with [^3H]thymidine for 8 h and harvested at the indicated times for determination of acid-precipitable radioactivity.

into stimulated-thymocyte glycopeptides was about 10-fold greater. The amount of radioactivity from [$2\text{-}^3\text{H}$]mannose incorporated into glycopeptides by quiescent thymocytes was barely detectable, making it difficult to determine whether or not there are qualitative differences in the glycopeptide species compared with stimulated thymocytes. The predominant peak of [$2\text{-}^3\text{H}$]mannose-labelled glycopeptides present in stimulated thymocytes occurs between fractions 29 and 31 and contains 40% of the total radioactivity incorporated into glycopeptides. The elution profile of [$2\text{-}^3\text{H}$]mannose-labelled glycopeptides synthesized by acute-leukaemic lymphoblasts is qualitatively similar to that for stimulated thymocytes, with almost 50% of the total radioactivity incorporated into glycopeptide eluted in a peak at fractions 29–31.

Quiescent thymocytes and concanavalin A-stimulated thymocytes were metabolically labelled with [$2\text{-}^3\text{H}$]mannose for a 12–24 h period. In Fig. 2(c), the elution profiles from chromatography on Bio-Gel P6 of the labelled glycopeptides present after 24 h are illustrated. The elution profiles are quantitatively and qualitatively similar to those obtained after the 0–12 h labelling period, indicating that biosynthetic

Fig. 2. Bio-Gel P6 elution profiles of $[2\text{-}^3\text{H}]$ mannose-labelled glycopeptides from quiescent thymocytes ($\bullet$), concanavalin A-stimulated thymocytes ($\circ$) and acute-leukaemic lymphoblasts ($\triangle$) after 0–12 h labelling period (a, b) and 12–24 h labelling period (c)

Arrows indicate the elution of molecular-weight markers (Blue Dextran, fetuin oligosaccharide, desialylated fetuin oligosaccharide, stachyose and mannose) in order of increasing fraction number.

capabilities have not altered. The amount of radioactivity incorporated into glycopeptide by quiescent thymocytes was about 3% of that by stimulated thymocytes.

$[6\text{-}^3\text{H}]$ Fucose incorporation into glycopeptides of quiescent thymocytes, concanavalin A-stimulated thymocytes and acute-leukaemic lymphocytes

All three cell types were metabolically labelled with $[6\text{-}^3\text{H}]$ fucose for 0–12 h, and quiescent thymocytes and concanavalin A-stimulated thymocytes for 12–24 h labelling periods. Concanavalin A was present from 0 h with the stimulated thymocytes. Before gel filtration on Bio-Gel P6, the labelled glycopeptides were treated with alkaline borohydride, as described in the Materials and methods section, to restrict the elution profile to *N*-glycosidically linked fucose-labelled glycopeptides. The elution profiles for glycopeptides labelled for 0–12 h are shown in Figs. 3(a) and 3(b).

Fucose incorporation into *N*-glycosidically linked glycopeptides resolved by chromatography on Bio-Gel P6 is both quantitatively and qualitatively similar in quiescent and concanavalin A-stimulated thymocytes. This result contrasts with the differences observed in mannose incorporation into glycopeptides of the two cell types over the same labelling period (Fig. 2a). The fucose-labelled glycopeptides are eluted as a poorly resolved mixture of two general apparent molecular-weight classes of 5300–4000 and 3300–2300. Fucose-labelled glycopeptides of acute-leukaemic lymphoblasts were not resolved into the same two apparent molecular-weight classes, but contained proportionally more of higher-molecular-weight glycopeptides than did either the quiescent or stimulated thymocytes.

The Bio-Gel P6 elution profiles of $[6\text{-}^3\text{H}]$ fucose-labelled glycopeptides synthesized by quiescent and concanavalin A-stimulated thymocytes (concanavalin A present from 0 h) over a 12–24 h labelling period are shown in Fig. 3(c). The patterns are similar to each other and to the elution profiles obtained after the 0–12 h labelling period (Fig. 3a); however, there were some low-molecular-weight structures found after the 12–24 h labelling period that were not present in the initial 12 h. Quiescent thymocytes were about 70% as active as concanavalin A-stimulated thymocytes in incorporating fucose into glycopeptides in the 12–24 h labelling period, though the extent of labelling indicates that both cell types are competent in synthesizing fucose-containing complex oligosaccharides.

Characterization of glycopeptides from Bio-Gel P6 chromatography: radioactive-sugar analysis

The amount of metabolic conversion of the radioactive sugar substrates, $[6\text{-}^3\text{H}]$ fucose and $[2\text{-}^3\text{H}]$ mannose, into other neutral monosaccharides

Fig. 3. Bio-Gel P6 elution profiles of $[6\text{-}^3\text{H}]\text{fucose}$ -labelled glycopeptides from quiescent thymocytes ($\bullet$), concanavalin A-stimulated thymocytes ($\circ$) and acute-leukaemic lymphoblasts ($\triangle$) after 0–12 h labelling period (a, b) and 12–24 h labelling period (c)

Arrows indicate molecular weight markers as in Fig. 2.

was assessed by paper-chromatographic identification of the radioactive sugar present after acid hydrolysis of individual peaks from Bio-Gel P6 chromatography. The peaks hydrolysed were selected from both $[6\text{-}^3\text{H}]\text{fucose}$ - and $[2\text{-}^3\text{H}]\text{mannose}$ -labelling experiments to represent a variety of apparent molecular weights, labelling periods and cell types. The low incorporation of $[2\text{-}^3\text{H}]\text{mannose}$ into glycopeptides by quiescent thymocytes prevented further analysis. In all three cell types

$[6\text{-}^3\text{H}]\text{fucose}$ was not metabolized to other neutral sugars (results not shown); however, $[2\text{-}^3\text{H}]\text{mannose}$ was converted, in varying amounts, into fucose (Table 1). Higher-molecular-weight glycopeptides from $[2\text{-}^3\text{H}]\text{mannose}$ -labelled cells had more radioactivity appearing in fucose, indicating a complex structure for these oligosaccharides. The major peak, fraction 30, for $[2\text{-}^3\text{H}]\text{mannose}$ -labelled concanavalin A-stimulated thymocytes and acute-leukaemic lymphoblasts was labelled exclusively with radioactive mannose.

Characterization of the glycopeptide (fraction 30) from $[2\text{-}^3\text{H}]\text{mannose}$ -labelled concanavalin A-stimulated thymocytes and acute-leukaemic lymphoblasts as containing a high-mannose oligosaccharide

The predominant peak present in the Bio-Gel P6 elution profiles of $[2\text{-}^3\text{H}]\text{mannose}$ -labelled glycopeptides from concanavalin A-stimulated and acute-leukaemic lymphoblasts was characterized as a high-mannose oligosaccharide by its ability to bind to immobilized concanavalin A (Fig. 4), sensitivity to endoglycosidase H and α -mannosidase and resistance to β -N-acetylglucosaminidase (Fig. 5). The affinity-chromatography and glycosidase-treatment data (Figs. 4 and 5) are presented for the characterization of fraction 30 from $[2\text{-}^3\text{H}]\text{mannose}$ -labelled acute-leukaemic lymphoblasts; identical results were obtained with fraction 30 from $[2\text{-}^3\text{H}]\text{mannose}$ -labelled concanavalin A-stimulated thymocytes.

All of the radioactivity present in fraction 30 bound to the column of immobilized concanavalin A and was quantitatively eluted at about 0.04 M α -methyl glucoside (Fig. 4). This affinity to concanavalin A indicates the presence of at least two α -mannosyl residues which are either terminal or substituted only at C-2 (Ogata *et al.*, 1975).

Endoglycosidase H is an endo- β -N-acetylglucosaminidase which requires the tetrasaccharide $\text{Man}_3\text{GlcNAc}$ for activity and is active on glycopeptides containing high-mannose-oligosaccharides (Kobata, 1979). Treatment of fraction 30 with endoglycosidase H and subsequent gel filtration on Bio-Gel P6 resulted in a shift of the peak to fraction 36. Since some ovalbumin glycopeptides containing non-reducing terminal N-acetylglucosamine residues are sensitive to endoglycosidase H (Kobata, 1979), glycopeptide from fraction 30 was treated with α -mannosidase and β -N-acetylglucosaminidase to determine whether the non-reducing terminal sugars were mannose or N-acetylglucosamine. Bio-Gel P6 chromatography after glycosidase treatments (Fig. 5) indicated that fraction 30 was insensitive to β -N-acetylglucosaminidase, but was almost entirely hydrolysed to radioactive free mannose by α -mannosidase. These results suggest that the glycopeptide, fraction 30, from $[2\text{-}^3\text{H}]\text{mannose}$ -

Table 1. Conversion of [2-³H]mannose into other neutral monosaccharides

Glycopeptides were prepared from concanavalin A-stimulated thymocytes and acute-leukaemic lymphoblasts metabolically labelled with [2-³H]mannose. Individual glycopeptide fractions from a Bio-Gel P6 column were hydrolysed to determine the amount of radioactivity present in other neutral sugars, as described in the Materials and methods section.

Cell type and labelling period	Glycopeptide fraction no.	Radioactivity (%)			
		Gal	Glc	Man	Fuc
Concanavalin A-stimulated thymocytes, 0–12 h	21	0	0	78	22
	26	0	0	82	18
	30	0	0	100	0
Concanavalin A-stimulated thymocytes, 12–24 h	22	0	0	36	64
	24	0	0	42	58
	30	0	0	100	0
Acute-leukaemic lymphoblasts, 0–12 h	21	0	0	70	30
	24	0	0	64	36
	31	0	0	100	0

Fig. 4. Concanavalin A affinity chromatography of glycopeptide eluted in fraction 30 from Bio-Gel P6 column of [2-³H]mannose-labelled acute-leukaemic lymphoblasts

Arrow indicates fraction at which sample was loaded on the column; Δ , ³H; ----, [α -methyl glucoside]. For full details see the Materials and methods section.

labelled concanavalin A-stimulated thymocytes and acute-leukaemic lymphoblasts contains a high-mannose oligosaccharide consisting of seven or eight mannose residues on a core di-N-acetylchitobiosyl unit linked N-glycosidically to asparagine.

Pulse/chase labelling of concanavalin A-stimulated thymocytes and acute-leukaemic lymphoblasts with [2-³H]mannose

The biosynthesis of both complex and high-mannose oligosaccharides which are N-glycosidically linked to protein proceeds through a common

Fig. 5. Bio-Gel P6 elution profiles of glycopeptide eluted in fraction 30 from [2-³H]mannose-labelled acute-leukaemic lymphoblasts after treatment with (a) endo-glycosidase H, (b) α -mannosidase or (c) β -N-acetylglucosaminidase

Arrows indicate molecular-weight markers as in Fig. 2. For full details see the Materials and methods section.

precursor, Glc₃Man₉GlcNAc₂. This oligosaccharide is processed by the sequential action of specific glycosidases and glycosyltransferases to form the desired structure (Robbins *et al.*, 1977). Since a precursor-product relationship between high-mannose oligosaccharides and complex oligosaccharides has been observed in metabolic labelling experiments in other systems, such as intestinal epithelial cells (Herscovics *et al.*, 1980), pulse/chase

Fig. 6. Bio-Gel P6 elution profiles of $[2\text{-}^3\text{H}]\text{mannose}$ -labelled glycopeptides from concanavalin A-stimulated thymocytes (O) and acute-leukaemic lymphoblasts (Δ) after a 0–12 h pulse and 12–24 h chase periods. Arrows indicate molecular-weight markers as in Fig. 2.

experiments were performed with stimulated thymocytes and acute-leukaemic lymphoblasts to determine whether the high-mannose glycopeptide, fraction 30, was an intermediate in the biosynthesis of complex oligosaccharides or a completed product of this pathway. Cells were labelled with $[2\text{-}^3\text{H}]\text{mannose}$ for the initial 12 h pulse period and placed in fresh media for the subsequent 12 h chase period. Glycopeptides were prepared at the end of the chase period and chromatographed on Bio-Gel P6. The elution profiles (Fig. 6) of the $[2\text{-}^3\text{H}]\text{mannose}$ -labelled glycopeptides for both stimulated thymocytes and acute-leukaemic lymphoblasts are very similar to those observed with these cells after 12 h (Figs. 2a and 2b). The major peak in both profiles, fraction 30, was characterized by concanavalin A affinity chromatography, sensitivity to endoglycosidase H and α -mannosidase as well as resistance to β -N-acetylglucosaminidase as being identical with the high-mannose glycopeptide present after 12 h. The presence of the high-mannose glycopeptide at the end of the chase period indicates that it is a completed product rather than an intermediate destined for further processing.

Discussion

Quiescent thymocytes, mitogen-stimulated thymocytes and acute-leukaemic lymphoblasts provide a model for the study of the biosynthesis of glycoproteins and their expression in quiescent cells, mitotically activated non-malignant and malignant cells respectively. The metabolic incorporation of $[2\text{-}^3\text{H}]\text{mannose}$ into N-glycosidically linked glycoproteins monitors the biosynthesis of both complex and high-mannose oligosaccharides, whereas $[6\text{-}^3\text{H}]\text{fucose}$ labels only the former class.

The biosynthesis of fucosylated complex oligosaccharides is both quantitatively and qualitatively similar in quiescent and stimulated thymocytes over 0–12 h (Fig. 3a) and 12–24 h (Fig. 3c) labelling periods, with quiescent thymocytes being about 70% as active as stimulated thymocytes in the 12–24 h period. The fucosylated glycopeptides were poorly resolved by Bio-Gel P6 chromatography, but appeared to consist of two broad molecular-weight groups, 5300–4000 and 3300–2300. The resolving power of gel-exclusion chromatography does not permit estimations of the number of different fucosylated glycopeptides present in the elution profile; however, a detailed study by Blithe *et al.* (1980) of fucose-labelled glycopeptides of BHK fibroblasts indicates the presence of about 40 different structures. Thus there may be differences in structure of complex oligosaccharides synthesized by quiescent and stimulated thymocytes which are not detected by gel-exclusion chromatography.

Considerable differences were observed in the biosynthesis of $[2\text{-}^3\text{H}]\text{mannose}$ -labelled glycopeptides by quiescent and stimulated thymocytes (Figs. 2a and 2c). Stimulated thymocytes incorporated over 10-fold more $[2\text{-}^3\text{H}]\text{mannose}$ into glycopeptide than did quiescent thymocytes in both 0–12 h and 12–24 h labelling periods. The low incorporation by quiescent thymocytes is not due to a generally inactive protein-glycosylation pathway, since fucosylation of glycoproteins is actively occurring during the same labelling periods (Figs. 3a and 3c). The increased incorporation of $[2\text{-}^3\text{H}]\text{mannose}$ into glycopeptide appears to be a specific response to mitogenic stimulation. The major glycopeptide peak from Bio-Gel P6 chromatography, fraction 30 (Figs 2a and 2c), present in stimulated thymocytes was characterized by Bio-Gel P6 and concanavalin A affinity chromatography (Fig. 4), endoglycosidase H and α -mannosidase sensitivity and β -N-acetylglucosaminidase resistance (Fig. 5), as a high-mannose-oligosaccharide-containing glycopeptide with an oligosaccharide of $\text{Man}_{7-8}\text{GlcNAc}_2$. Although this glycopeptide contained about 40% of the radioactivity in $[2\text{-}^3\text{H}]\text{mannose}$ -labelled stimulated thymocytes, it is not possible to argue that on a mass basis it is the dominant mannose-containing structure formed, because the high content of this sugar is likely to ensure a greater amount of labelling than an oligosaccharide containing fewer mannose residues.

Similarities in structure between the high-mannose oligosaccharide formed in fraction 30, $\text{Man}_{7-8}\text{GlcNAc}_2$, and intermediates in the biosynthesis of complex oligosaccharides suggested a possible precursor role for the high-mannose oligosaccharide formed on mitogenic stimulation. Pulse/chase experiments (Fig. 6) show that this same structure is present after a 12–24 h chase period, a period in which the stimulated thymocytes are

competent in both fucose and mannose incorporation into glycopeptide, and probably represents an end product of the biosynthetic pathway rather than an intermediate to complex oligosaccharide structures.

The [^3H]thymidine-incorporation data (Fig. 1) indicate that the concanavalin A-stimulated thymocytes do not enter the S phase of the cell cycle until after 24 h exposure to the mitogen. The synthesis of the high-mannose oligosaccharide occurs within the first 12 h of mitogenic stimulation preceding mitosis. The ability of the mitogen, concanavalin A, to switch on the synthesis of high-mannose oligosaccharides by thymocytes provides a model for studying the regulation of protein glycosylation. The transferrin receptor, a membrane glycoprotein expressed on a cultured leukaemia cell line (Trowbridge & Omary, 1981), has also been demonstrated to be synthesized in thymocytes only upon mitogenic stimulation and before DNA synthesis (Larrick & Cresswell, 1979).

[$6\text{-}^3\text{H}$]Fucose labelling of acute-leukaemic lymphoblasts resulted in a shift towards higher-molecular-weight fucosylated glycopeptides relative to those of quiescent and stimulated thymocytes which had similar labelling patterns (Figs. 3a and 3b). This observation is consistent with the fucose-labelling pattern of human quiescent and stimulated peripheral lymphocytes and leukaemic cells (Van Beek *et al.*, 1975). [$2\text{-}^3\text{H}$]Mannose labelling of acute-leukaemic lymphoblasts resulted in a glycopeptide pattern which was qualitatively similar to that of concanavalin A-stimulated thymocytes (Figs. 2a and 2b), with the fraction from Bio-Gel P6 chromatography having the most radioactivity being characterized (Figs. 4 and 5) as a glycopeptide containing the high-mannose oligosaccharide, $\text{Man}_{7-8}\text{GlcNAc}_2$. Pulse/chase experiments (Fig. 6) indicate that in the leukaemic cells, as in the mitogen-stimulated thymocytes, the high-mannose structure is an end product of the biosynthetic pathway.

The pattern of incorporation of [$6\text{-}^3\text{H}$]fucose and [$2\text{-}^3\text{H}$]mannose into glycopeptides of quiescent thymocytes, mitogen-stimulated thymocytes and acute-leukaemic lymphoblasts indicates that an increase in molecular weight of fucose-labelled glycopeptides is characteristic of the malignant cells, whereas mitotically active cells, both malignant and non-malignant, specifically synthesize more of a high-mannose oligosaccharide tentatively identified as $\text{Man}_{7-8}\text{GlcNAc}_2$.

C. A. R. is a recipient of a post-doctoral fellowship from the Medical Research Council of Canada and G. M. W. C. is a Member of the External Scientific Staff, Medical Research Council, U.K. We thank Miss M. J. Barber for excellent technical assistance.

References

- Atkinson, P. H. & Hakimi, J. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 191–239, Plenum Press, New York and London
- Baker, S. R., Blithe, D. L., Buck, C. A. & Warren, L. (1980) *J. Biol. Chem.* **255**, 8719–8728
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Blithe, D. L., Buck, C. A. & Warren, L. (1980) *Biochemistry* **19**, 3386–3395
- Buck, C. A., Glick, M. C. & Warren, L. (1971) *Biochemistry* **10**, 2176–2180
- Ceccarini, C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2687–2690
- Ceccarini, C. & Atkinson, P. H. (1977) *Biochim. Biophys. Acta* **500**, 197–207
- Ceccarini, C., Muramatsu, T., Tsang, J. & Atkinson, P. H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3139–3143
- Cook, G. M. W. & Jacobson, W. (1968) *Biochem. J.* **107**, 549–557
- Davidson, W. & Parish, C. R. (1975) *J. Immunol. Methods* **7**, 291–300
- Herscovics, A., Bugge, B., Quaroni, A. & Kirsch, K. (1980) *Biochem. J.* **192**, 145–153
- Kobata, A. (1979) *Anal. Biochem.* **100**, 1–14
- Larrick, J. W. & Cresswell, P. (1979) *J. Supramol. Struct.* **11**, 579–586
- Li, Y. T. & Li, S. C. (1972) *Methods Enzymol.* **28**, 702–713
- Mizuochi, T., Yonemasu, K., Yamashita, K. & Kobata, A. (1978) *J. Biol. Chem.* **253**, 7404–7409
- Muramatsu, T., Koide, N., Ceccarini, C. & Atkinson, P. H. (1976) *J. Biol. Chem.* **251**, 4673–4679
- Nilsson, B., Nordén, N. E. & Svensson, S. (1979) *J. Biol. Chem.* **254**, 4545–4553
- Ogata, S., Muramatsu, T. & Kobata, A. (1975) *J. Biochem. (Tokyo)* **78**, 687–696
- Partridge, S. M. (1949) *Nature (London)* **164**, 443
- Robbins, P. W., Hubbard, S. C., Turco, S. J. & Wirth, D. F. (1977) *Cell* **12**, 893–900
- Spiro, R. G. (1966) *Methods Enzymol.* **8**, 3–26
- Spiro, R. G. & Bhoyroo, V. D. (1974) *J. Biol. Chem.* **249**, 5704–5717
- Trowbridge, I. S. & Omary, M. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3039–3043
- Van Beek, W. P., Smets, L. A. & Emmelot, P. (1975) *Nature (London)* **253**, 457–460
- Warren, L., Buck, C. A. & Tuszyński, G. P. (1978) *Biochim. Biophys. Acta* **516**, 97–127