THE RIBOSOMAL INCORPORATION OF HEXOSAMINE INTO GLYCOPROTEIN IN A MOUSE MYELOMA

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Received 28 March 1970

1. Introduction

In a variety of tissues the bulk of hexosamine incorporation into glycoprotein occurs in the microsomal membranes [1]. Several studies have suggested that the addition of a small proportion of the total carbohydrate can occur while the glycoprotein polypeptide is still associated with the ribosome [2–5], but these results are difficult to evaluate since it has been shown that polyribosomes prepared from rat liver can become contaminated with non-ribosomal proteins [6, 7]. In this study the IgA-secreting mouse plasmacytoma MPC-1 was used in experiments designed to differentiate clearly between isotopic labelling due to the incorporation of hexosamine into nascent glycoprotein at the ribosomal site and labelling caused by contamination of the preparation by non-ribosomal proteins. These experiments indicate that the ribosomal labelling observed represents the sum of two contributing factors: the incorporation of hexosamine into nascent glycoprotein and contamination by non-ribosomal protein. A part of this study has been previously published as a preliminary communication [8].

2. Methods

The MPC-1 plasmacytoma was maintained by serial transplantation in Balb/c mice. Tumour tissue was removed from decapitated mice, washed twice in 0.15 M NaCl, the cells dispersed by gentle squeezing through a single layer of surgical gauze and washed in Eagle's medium [9]. The cells were resuspended in Eagle's medium containing 50 mM Na pyruvate, 5% v/v horse serum and 10 μCi of 1-^{14}C-glucosamine (specific activity 55 mCi/mmole), and subsequently incubated in stoppered flasks at 37° in an O_2/CO_2 (95:5%) atmosphere. Incubations were terminated by chilling to 0°. Puromycin was added to the incubation flasks at various times during some of the experiments to a final concentration of 1 mM; these incubations were continued for a further 10 min. Cells recovered from incubation media by centrifugation were homogenized in 8 ml of 0.25 M buffered sucrose [10] and the homogenate centrifuged at 10,000 g (R_{ave}) for 10 min. The supernatant was adjusted to a final deoxycholate concentration of 1% with 10% sodium deoxycholate in 0.03 M tris-hydrochloric acid buffer, pH 8.3. This suspension was centrifuged at 13,500 g (R_{ave}) for 10 min, the supernatant layered over a discontinuous gradient as described by Robinson [7], and the tubes centrifuged at 102,000 g (R_{ave}) for 210 min; the supernatants were retained. The polyribosomal pellets were suspended in 0.25 M sucrose containing tris-hydrochloric acid buffer pH 7.4 (50 mM), KCl (25 mM) and $MgCl_2$ (5 mM) and gently homogenized. This suspension was centrifuged at 13,500 g (R_{ave}) for 10 min to remove undispersed material, and then at 105,000 g (R_{ave}) for 60 min to sediment the polyribosomes.

Polyribosomal pellets and their deoxycholate supernatants were prepared for counting by precipitation of protein with chilled 10% (w/v) trichloroacetic acid. The precipitates were washed twice with cold 10% (w/v) trichloroacetic acid, twice with chloroform: methanol: ether (1:1:2 v/v) and the precipitates hydrolysed in trichloroacetic acid (5% w/v, 15 min, 90°); the residues were recovered by centrifugation. The supernatants were analyzed

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for their ribose contents according to Schneider [11], and the residues dissolved in 0.5 N NaOH at 80°. These solutions were used for protein determination [12] and radioactivity measurements by scintillation counting. Analysis using methods described by Robinson [13] showed glucosamine to be the only labelled substance present.

The specific activity of UDP-*N*-acetyl glucosamine was determined in tissue incubated for 20, 40 and 60 min. Incubated cells were homogenized in 20 ml of absolute ethanol at −10°, and the resulting precipitates were removed by centrifugation. The supernatants were reduced to dryness at room temperature in a rotary evaporator, the residues redissolved in water, and the nucleotides adsorbed onto 0.5 g of dried, acid-washed charcoal. The charcoal was isolated by filtration, and exhaustively eluted with 0.03 M NH_4OH in 50% ethanol. The combined eluates were evaporated, hydrolyzed in 3 ml of 10 N HCl (100°, 3 hr) and the acid removed by rotary evaporation at 60°. Hexosamines were isolated on Dowex-50 (H^+) columns and eluted with 2 N HCl; a portion of this eluate was retained for scintillation counting and the glucosamine content of the remainder was determined [14].

3. Results and discussion

The RNA/protein ratios for the polyribosomes prepared as described fell in the range 0.90–1.10. The linear incorporation of ^{14}C-glucosamine into polyribosomes and deoxycholate-soluble protein for incubation periods up to 90 min is shown in fig. 1. Addition of puromycin to the incubation medium at the times shown resulted in a sharp drop in the specific activity of the polyribosomes and a decline in the rate of increase of activity in the deoxycholate supernatant. If all the labelling observed represented incorporated hexosamine, then the addition of puromycin would discharge the growing polypeptides [15] and the specific activities of the polyribosomes would fall to similar low values irrespective of when puromycin was added. If on the other hand, the linear incorporation observed was solely due to the adsorption

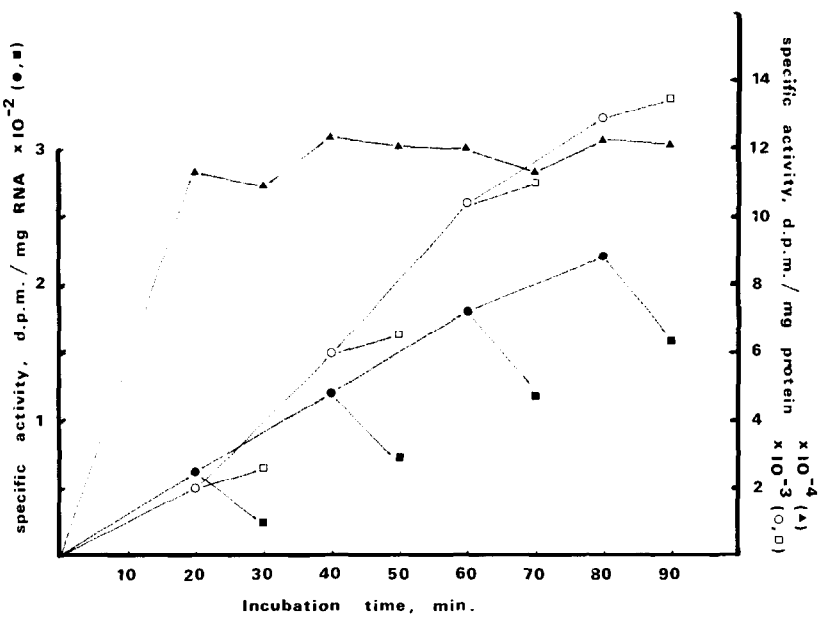

Fig. 1. Plasmacytoma tissue was incubated as described in pairs of flasks for 20, 40, 60 and 80 min. At the end of each incubation period, the control flask of each pair was withdrawn; puromycin was added to the other to a concentration of 1.0 mM. The puromycin-containing flasks were withdrawn after a further 10 min of incubation. Polyribosomes and deoxycholate supernatants were prepared from the incubated cells as described in the text.

● = control polyribosomes
■ = puromycin-treated polyribosomes
○ = control deoxycholate supernatants
□ = puromycin-treated deoxycholate supernatants
▲ = trichloroacetic acid supernatant.
↑ = time of puromycin addition.

of contaminating material, then the addition of puromycin would have an effect proportional only to the reduction in specific activity of the deoxycholate supernatant caused by the antibiotic. The actual decreases in the polyribosomal specific activities are disproportionately large when compared with the corresponding decreases observed in the radioactivities of the deoxycholate-soluble protein. This finding indicates that the reduction in ribosomal radioactivity as a result of puromycin addition is not wholely due to a decrease in the specific activity of the contaminating supernatant protein. Furthermore, no discharge of labelled material occurs when contaminated polyribosomes are incubated with puromycin *in vitro* [16]. Therefore the decreases in polyribosomal activities indicate the presence of ^{14}C-glucosamine-labelled nascent glycoprotein which is released from the polyribosomes by the puromycin. The extent of the puromycin-induced reduction in polyribosomal specific activity increases with progressively longer incubation times and reflects the slow rise to equilibrium of the radioactivity of the immediate precursor of incorporated glucosamine, i.e. UDP-*N*-acetyl glucosamine (table 1). The residual radioactivity in polyribosomes from puromycin treated cells probably represents contaminating protein, since the rate of increase with time of this residual radioactivity is proportional to the increase in the radioactivity of the deoxycholate supernatant protein (fig. 1).

It is concluded, therefore, that the trichloroacetic acid insoluble (i.e. protein bound) ^{14}C-glucosamine associated with polyribosomes prepared from these plasmacytoma cells is derived from both nascent glycoprotein and from contaminating supernatant glycoprotein. Recent observations [17] have also shown that glucosamine becomes attached to nascent glycoprotein in rat liver.

Table 1

Incubation time (min)	20	40	60
Specific activity of UDP-*N*-acetylglucosamine, (dpm/μmole X 10^{-6})	3.87	5.47	6.82
Incubation time, (min)	30	50	70
Drop in specific acitivity of polyribosomes due to the addition of puromycin (dpm/mg RNA)	47	60	78

Plasmacytoma tissue was incubated in the presence of ^{14}C-glucosamine as described in the text for 20, 40 and 60 min, and the specific activity of the intracellular UDP-*N*-acetyl glucosamine determined. The drop in the polyribosomal specific acitivities as a result of puromycin addition is calculated from the data of fig. 1; the figures are corrected for the corresponding reduction in radioactivity in the deoxycholate supernatant.

Acknowledgements

One of us (N.J.C.) is grateful to the Medical Research Council for a scholarship for training in research methods. We wish to thank Dr. B.A.Askonas for providing the tumour and the British Empire Cancer Campaign for financial help.

References

[1] R.G.Spiro, New Eng. J. Med. 281 (1969) 991.
[2] L.Helgeland, Biochim. Biophys. Acta 101 (1965) 106.
[3] J.Molnar, G.B.Robinson and R.J.Winzler, J. Biol. Chem. 240 (1965) 1882.
[4] G.R.Lawford and H.Schachter, J. Biol. Chem. 241 (1966) 5408.
[5] F.Melchers and P.M.Knopf, Cold Spring Harbour Symp. Quant. Biol. 32 (1967) 255.
[6] C.N.Murty and T.Hallinan, Biochem. J. 112 (1969) 269.
[7] G.B.Robinson, FEBS Letters 4 (1969) 190.
[8] N.J.Cowan and G.B.Robinson, Biochem. J. 115 (1969) 57P.
[9] H.Eagle, Science 130 (1959) 432.
[10] A.Korner, Biochem. J. 81 (1961) 168.
[11] W.C.Schneider, in: Methods in Enzymology, Vol. 3, eds. S.P.Colowick and N.O.Kaplan (Academic Press, New York, 1957) p. 680.
[12] O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.
[13] G.B.Robinson, Biochem. J. 108 (1968) 275.
[14] J.R.Clamp, G.Dawson and L.Hough, Biochim. Biophys. Acta 148 (1967) 342.
[15] D.Nathans, Federation Proc. 23 (1964) 984.
[16] G.B.Robinson, unpublished results.
[17] G.B.Robinson, Biochem. J. 115 (1969) 1077.