Cell surface expression of 4 β -galactosyltransferase accompanies rat parotid gland hypertrophy induced by changes in diet

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Maintenance of rats for 2 weeks on a diet consisting of 50% inert cellulose and 50% laboratory chow resulted in hypertrophy of the parotid gland and a 4-fold increase in total membrane-associated 4 β -galactosyltransferase enzyme activity (EC 2.4.1.38). Localization of the increased specific activity to the cell surface of the enlarged gland was shown by subcellular fractionation of Golgi and plasma membranes. This observation was confirmed by enzyme assays of intact cells; quantification of immunofluorescence was made by using a fluorescence activated cell sorter. Parotid gland hypertrophy was inhibited by the administration of the specific modifier protein α -lactalbumin as well as by a monospecific antibody for 4 β -galactosyltransferase. These agents also inhibited the incorporation of thymidine into DNA.

INTRODUCTION

Membrane-associated glycosyltransferases have classically been localized to the Golgi apparatus where these enzymes function in post-translational modification of proteins and lipids (Kornfeld & Kornfeld, 1985). Additional evidence continues to accumulate suggesting that a portion of the cellular activity of glycosyltransferases is present at the cell surface and oriented towards the extracellular space (Roseman, 1970; Shur, 1984; Roth *et al.*, 1985).

Galactosyltransferases at the surfaces of contacting cells were initially implicated as a possible mediator of cell growth by Roth & White (1972). Since then various differences in 4 β -galactosyltransferase activity between normal and transformed cells have been reported (Bernacki & Kim, 1977; Roth *et al.*, 1977), and enzyme levels in serum (Bossman & Hall, 1974; Ip & Dao, 1977) or variant isoenzymes (Podolsky & Weiser, 1975) of the transferase in humans have been correlated with the presence of various tumours.

Isoprenaline, a β -adrenergic agonist, has a dramatic growth-stimulating effect on the parotid glands of rats and mice. Chronic administration of this agent leads to hyperplastic and hypertrophic enlargement (Selye *et al.*, 1961; Brown-Grant, 1961; Barka, 1965; Schneyer, 1962, 1972). Gland hyperplasia is most pronounced during the initial phase of this treatment, but is not, however, neoplastic, for it results in a limited enlargement that is partially reversible upon cessation of drug treatment (Selye *et al.*, 1961; Schneyer, 1962). In addition, isoprenaline causes a dramatic increase in 4 β -galactosyltransferase in the plasma membranes of the hypertrophied parotid gland (Humphreys-Beher, 1987) and also results in the active secretion of the enzyme into saliva (Humphreys-Beher, 1984). The increase in enzyme activity has been shown to be due to an increase in gene expression (Humphreys-Beher, 1984; Humphreys-Beher *et al.*, 1986). The introduction of the enzyme modifier protein, α -lactalbumin, specific substrates of 4 β -galactosyltransferase (ovalbumin), or a monospecific antiserum to the transferase (Humphreys-Beher, 1984), when

incorporated into the isoprenaline drug regimen, have been shown to inhibit the effects of isoprenaline on parotid gland hypertrophy (Humphreys-Beher, 1987).

Hypertrophic and hyperplastic growth of rat parotid glands also occurs in response to more physiological conditions than those associated with isoprenaline. One such system involved provision to animals of a diet consisting of 50% ground chow and 50% inert cellulose (bulk diet) (Hall & Schneyer, 1977, 1978). In order for animals to consume adequate amounts of food, the amount of take-in is increased, prolonging the masticatory period. Thus our investigations have previously shown the induction of proline-rich proteins and increased 4 β -galactosyltransferase enzyme activity in parotid glands enlarged following introduction of this diet (Humphreys-Beher *et al.*, 1987). In the present work, we have sought evidence for an alteration in the subcellular localization of the increased 4 β -galactosyltransferase to the plasma membrane. In addition, the introduction of specific substrates and modifier proteins for 4 β -galactosyltransferase with the bulk-diet inhibit transferase-mediated cell growth.

MATERIALS AND METHODS

Materials

Ovalbumin, UDP-galactose, UDP-glucose, GDP-mannose, collagenase, UTP, UMP, UDP, hyaluronidase, bovine submaxillary mucin and bovine α -lactalbumin (Ca²⁺-depleted) were from Sigma Chemical Co. UDP-[¹⁴C]galactose (sp. radioactivity 300 mCi/mmol) was obtained from New England Nuclear. [6-³H]Thymidine (6.7 Ci/mmol) was purchased from Amersham International. Rats (Long-Evans strain) were purchased from Charles River Breeding Farms. The monospecific nature and species cross-reactivity of the 4 β -galactosyltransferase antibody have previously been established (Humphreys-Beher, 1984; Humphreys-Beher *et al.*, 1986). All other reagents were purchased through commercial sources and were of reagent grade quality.

Preparation of isolated parotid gland acinar cells and membranes

Male Long-Evans strain rats weighing 175–225 g were fed a diet consisting of 50% ground Purina laboratory chow and 50% inert cellulose (bulk diet) for 2 weeks. The introduction of effectors of 4 β -galactosyltransferase was concomitant with the diet and they were administered by twice-daily intraperitoneal injections of the specific compounds in 0.5 ml of phosphate-buffered saline, pH 7.0. Food and water were provided *ad libitum* for 21 days. Parotid glands were identified by gross morphology following anaesthesia with pentobarbital and death by exsanguination. Intact cells were freed from connective tissue and dissociated with collagenase and hyaluronidase as described by Oliver *et al.* (1986).

Alternatively, total membrane fractions were prepared at 4 °C by homogenization of intact parotid tissue from either treated or control animals in 10 mM-Tris/HCl buffer, pH 8.0, with a Dounce apparatus. A low speed centrifugation at 500 *g* was then performed to remove connective tissue as well as unlysed cells. The resulting slurry was then centrifuged at 100 000 *g* for 1 h to recover total membrane. Protein assays were performed by a modification of the Lowry method with bovine serum albumin as the standard (Schachterle & Pollack, 1973). Plasma membranes were isolated by the procedure of Arvan & Castle (1982), a protocol designed specifically to isolate plasma membranes from rat parotid gland. The total membrane pellet was resuspended in 0.5 mM-MgCl₂/1 mM-NaHCO₃, pH 7.4, containing 0.7 mM-EDTA. The membrane slurry was centrifuged at 875 *g* for 15 min and the pellet resuspended in 0.5 vol. of the above buffer. The suspension was overlaid onto 5 ml of the same buffer containing 0.3 M-sucrose and recentrifuged at 12 500 *g* for 15 min. The pellet was adjusted to 1.38 M-sucrose (125 ml), overlaid with 0.3 M-sucrose, and centrifuged for 2 h in a Beckman swinging bucket rotor SW27 at 50 000 *g*. Plasma membranes were removed from the interface, placed in a solution containing 0.35 M-sucrose in 0.5 mM-MgCl₂/1 mM-NaHCO₃ (pH 7.4) containing 1.7 mM-EDTA, then centrifuged in an SW41 rotor for 2 h at 150 000 *g*. Fractions enriched for Golgi membranes were obtained from the supernatant of the 12 500 *g* centrifugation (Arvan & Castle, 1982). This supernatant was diluted to 12 ml in 0.3 M-sucrose and centrifuged at 100 000 *g* for 1 h. The resulting pellet is enriched for Golgi membranes. The final membrane pellets were resuspended in 10 mM-Tris buffer, pH 8.0, for subsequent assays for protein and 3 β - and 4 β -galactosyltransferase activities.

Galactosyltransferase assays

The activity of 3 β - and 4 β -galactosyltransferase was measured as previously described by Humphreys-Beher (1984). In brief, membrane fractions were obtained as described above and resuspended in 10 mM-Tris/HCl, pH 8.0, to give a final protein concentration of 200 μ g/ml. The assay mixture (total volume 50 μ l) contained 0.1 M-Mes (pH 6.3), 25 mM-MnCl₂, 0.5% Triton X-100, 1 mM-UDP-[1-¹⁴C]galactose (2 mCi/mmol), 1.0 mM-ovalbumin (for 4 β -) or 10 mM-asialo-bovine submaxillary mucin (for 3 β -) as acceptor and 0–0.5 mg of the membrane preparation. After incubation at 37 °C for 1 h the reaction was terminated by the addition of 1 ml of ice-cold 10% (w/v)

trichloroacetic acid and the precipitate was recovered on glass fibre filters.

Cell surface 4 β -galactosyltransferase assay

Intact cells from parotid glands of rats fed either bulk or chow diet were isolated by the protocol described above and washed into Ca²⁺ + Mg²⁺-free Hanks solution containing 10 mM-MnCl₂. The cells were resuspended in the same buffer and approximately 10⁶ cells were added to incubation tubes that also contained 200 μ M-UDP-[¹⁴C]galactose in a final volume of 50 μ l. Some tubes had no additions while others contained ovalbumin (10 mg/ml final concentration) in order to assess activity towards endogenous and exogenous acceptors, respectively. Enzyme assays were performed at 37 °C for 1 h with intermittent mixing of the cells. Lactate dehydrogenase assays on post-incubation supernatants and sonicated cells were performed to provide an estimate of enzyme leakage (Schnaar *et al.*, 1978). The reactions were terminated by the addition of 1 ml of ice-cold 10% (w/v) trichloroacetic acid and the incorporation of [¹⁴C]galactose was determined by scintillation counting following precipitation onto glassfibre filters. Zero-time controls were subtracted from all experimental values. Enzyme specific activities are expressed as nmol of galactose incorporated/min per mg of membrane protein.

Cell surface immunostaining

Dissociated cells for immunofluorescent studies of intact non-permeabilized cells (250 μ l of packed cells) were washed into 10 ml of a balanced salt solution containing 5% fetal calf serum and 0.02% NaN₃ (included to prevent energy-requiring rearrangements of cell surface constituents). The IgG fractions from the anti-4 β -galactosyltransferase serum or a pre-immune serum were added at a concentration corresponding to a 1:20 dilution of the initial serum. The incubation was carried out on ice for 30 min with periodic resuspension. The cells were subsequently washed three times in a 10 ml volume of the calf serum containing buffer and collected by centrifugation at 500 *g* for 10 min at 4 °C. Commercially available fluorescein isothiocyanate-conjugated anti-rabbit IgG antiserum (1:20) was then added to the cells, and following incubation and washing as described above they were fixed with 2% formalin for cell sorting.

A Becton-Dickerson FACStar Fluorescence-Activated Cell Sorter with a 2 W argon ion laser tuned to 488 nm was used to quantify cell surface labelling of 4 β -galactosyltransferase of cells from control and from enlarged parotid glands (Loken & Stall, 1982). Routinely 10 000 cells per condition were quantified for immunofluorescence.

Incorporation of [³H]thymidine into DNA

DNA synthesis *in vivo* in parotid glands of rats fed chow or bulk diets was followed by monitoring the incorporation of [³H]thymidine into trichloroacetic acid-precipitable counts. Injections of 10 mM- α -lactalbumin using the regimen described above were followed by the intraperitoneal administration of 1 μ Ci of [³H]thymidine after 7 days maintenance of rats on the bulk diet. The animals were killed 5 h following this injection, their glands removed and homogenized at 4 °C. Samples (100 μ l) were removed for precipitation

with trichloroacetic acid on glassfibre filters, followed by scintillation counting using Amersham premixed non-aqueous scintillation cocktail for ^3H incorporation. Part of the sample was removed for a protein assay. Duplicates of untreated animals were used to determine basal rates of DNA synthesis.

RESULTS AND DISCUSSION

Present data confirm previous observations that a diet consisting of 50% inert cellulose plus 50% ground chow induces a 2–3-fold enlargement of rat parotid gland and a 4-fold increase in total membrane-associated 4 β -galactosyltransferase (Humphreys-Beher *et al.*, 1987, and Table 1).

The specific activity of a second Golgi-associated galactosyltransferase, 3 β -galactosyltransferase (no EC number) remained unchanged. Subcellular fractionation of membrane compartments was used to determine the localization of the increased 4 β -galactosyltransferase activity. It was subsequently determined that membranes enriched for Golgi apparatus (Arvan & Castle, 1982) exhibited no changes in either enzyme's specific activity following the bulk diet regimen (Table 1). However, plasma membrane fractions prepared from parotid glands of bulk diet-treated animals showed a 50-fold stimulation in 4 β -galactosyltransferase specific activity while 3 β -galactosyltransferase activity remained low. The resulting specific activity for the 4 β -galactosyltransferase in the bulk-diet plasma membranes was nearly two-thirds that of the Golgi-enriched fractions.

In order to further investigate the enhancement in 4 β -galactosyltransferase activity observed with the bulk diet leading to gland hypertrophy, intact parotid acinar cells were isolated from enlarged and normal parotid glands and assayed for surface enzyme activity. As shown by data in Table 2, the incorporation of [^{14}C]galactose into endogenous acceptors by intact cells increased nearly 3-fold after introduction of a bulk diet. When the exogenous acceptor ovalbumin was present the increase was even more pronounced. This level of incorporation of radiolabel could not be attributed to cell breakage and the release of 4 β -galactosyltransferase into the medium, since assays for solubilized transferase following a mock incubation were negative and since leakage of lactate dehydrogenase was only 3% of the activity released by sonic disruption of the cells. Incorporation of [^{14}C]galactose could not be attributed

Table 2. Cell surface 4 β -galactosyltransferase assays of intact parotid cells

Treatment	^3H Galactose transferred to macromolecular acceptors (c.p.m.)*	
	Chow	Bulk
Endogenous acceptors only	1100 $\pm$ 200	3200 $\pm$ 100
Ovalbumin (10 mg/ml)	2700 $\pm$ 100	9700 $\pm$ 250
Ovalbumin + unlabelled UDP-Gal (2 mM)	150 $\pm$ 15	220 $\pm$ 20
Ovalbumin + unlabelled Gal (2 mM)	2900 $\pm$ 150	10300 $\pm$ 175
Ovalbumin + unlabelled AMP† (2 mM)	2700 $\pm$ 200	9900 $\pm$ 150
Leaked activity toward ovalbumin in supernatant‡	50 $\pm$ 10	200 $\pm$ 25
Sonicated cell activity towards ovalbumin	25 500 $\pm$ 250	68 200 $\pm$ 250

* Cells were removed by centrifugation following a mock assay. The supernatant was then assayed for leaked transferase. Values are expressed as the mean $\pm$ S.D. for three experimental determinations.

† A pyrophosphatase inhibitor.

‡ Cells were removed by centrifugation following a mock assay. The supernatant was then assayed for leaked transferase.

to internal utilization of the labelled material, since the inclusion of excess unlabelled galactose had no effect on incorporation while excess unlabelled UDP-galactose significantly diluted macromolecular label. These results suggest again the presence of 4 β -galactosyltransferase on the cell surface and also suggest that the enzyme activity is oriented on the exterior of the plasma membrane. Since it has previously been shown that cell membranes are impermeable to sugar nucleotides (Perez & Hirschberg, 1985), from the above results it may be inferred that the utilization of radiolabelled sugar was due to externally localized galactosyltransferase.

The differences observed in 4 β -galactosyltransferase activity between plasma membrane preparation and intact cell assays is not entirely clear. The addition of non-ionic detergent to the plasma membrane preparation enzyme assays may impart a higher specific activity than would otherwise be indicated. The addition of detergent to membrane preparations, in glycosyltransferase assays,

Table 1. Galactosyltransferase activities in parotid membrane fractions

Membrane fraction	4 β -Galactosyltransferase*		3 β -Galactosyltransferase†	
	Chow	Bulk§	Chow‡	Bulk§
Total membranes	0.25 $\pm$ 0.05	0.91 $\pm$ 0.19	1.89 $\pm$ 0.25	2.01 $\pm$ 0.20
Golgi-enriched	4.72 $\pm$ 0.23	4.89 $\pm$ 0.31	8.09 $\pm$ 0.33	7.82 $\pm$ 0.37
Plasma membranes	0.05 $\pm$ 0.03	2.63 $\pm$ 0.12	0.02 $\pm$ 0.02	0.03 $\pm$ 0.02

* Values are expressed as nmol/min per mg of protein utilizing ovalbumin (10 mg/ml) as an exogenous acceptor. The results represent the mean S.D. of three separate experimental determinations performed in duplicate.

† Values are expressed as nmol/min per mg of protein utilizing asialo-bovine submaxillary mucin (10 mg/ml) as exogenous acceptor.

‡ Chow diet refers to pellet laboratory chow.

§ Bulk diet refers to 50% ground pellet chow/50% inert cellulose.

has previously been shown to increase the specific activity of these enzymes (Jentoft *et al.*, 1976). This action may be due to solubilization of the enzyme, thereby increasing the frequency of contact with substrate molecules in the reaction mixture.

Isolated parotid acinar cells were also assessed for surface 4β -galactosyltransferase by indirect immunofluorescence using a monospecific rabbit antibody to 4β -galactosyltransferase (Humphreys-Beher, 1984) and a fluorescein-labelled secondary antibody. The result on non-permeabilized cells correlated well with the enzyme assays and served to confirm further the surface expression of this enzyme. A fluorescence-activated cell sorter was used to quantify the results of the immunofluorescence (Loken & Stall, 1982). At 48 h after

Fig. 1. Numbers of intact cells (expressed as response) displaying various levels of fluorescence following staining with anti- 4β -galactosyltransferase antibody and a fluorescein isothiocyanate-labelled secondary anti-rabbit antibody

Acinar cells isolated from chow-fed animals displayed two fluorescence levels of less than 6 and 10 log fluorescence units measured at 488 nm using the fluorescence-activated cell sorter (a). At 7 days of the bulk diet the majority of the parotid acinar cells have shifted to a single peak of 10–20 log fluorescence units (b). This level of surface fluorescence was observed over the 14 days of the bulk-diet regimen. The area under the two peaks in (a) is approximately equal to the area under the peak in (b), indicating that equivalent numbers of cells were assayed in the experiments.

initiation of the bulk diet regimen, mean fluorescence due to 4β -galactosyltransferase on the cell surface increased; it remained elevated during the 14 days of the regimen of the bulk diet (results not shown). Two populations of cells were apparent in the chow-fed control animals (Fig. 1a). The majority of cells isolated from parotid glands of animals on bulk diet was shifted to a higher level of fluorescence (Fig. 1b), and remained in this state throughout the period when bulk diet was fed. The level of surface 4β -galactosyltransferase on the cells showing the higher fluorescence would be expected if cell surface enzyme were involved in the transition to a growth phase in parotid acinar cells. The presence of two cell populations of low and high fluorescence in control animals indicates that the majority of cells are in a quiescent state, with a low level of surface 4β -galactosyltransferase. The fraction of the cells which show a higher level of fluorescence indicates that a certain amount of cell growth is taking place in the gland. The observation of the multiple cell population staining with anti-galactosyltransferase antibody has previously been described by Berger and co-workers (Berger *et al.*, 1981; Davis *et al.*, 1984; Jassin *et al.*, 1985) for a number of different cell types.

Parotid gland enlargement as a consequence of chronic isoprenaline was found to be inhibited in a dose-dependent manner by the specific modifier protein α -lactalbumin and by the substrates ovalbumin and UDP-galactose (Humphreys-Beher, 1987). To test the concept that 4β -galactosyltransferase is responsible in part for mediating gland hypertrophy in the animals fed the bulk diet, effectors of 4β -galactosyltransferase also were employed during the feeding of the bulk diet. Commercial preparations of α -lactalbumin were purified by C_1 -reversed phase column h.p.l.c. to ensure removal of minor protein contaminants and buffered salts. As shown by the data in Table 3, specific effectors of 4β -galactosyltransferase such as UDP-galactose and α -lactalbumin were able to prevent parotid gland hypertrophy in a dose-dependent fashion when injected intraperitoneally during the course of the bulk-diet regimen. The inhibition of gland hypertrophy was also evident by the retardation of [3 H]thymidine incorporation into DNA. The introduction of nucleotide sugars other than UDP-galactose did not inhibit parotid gland hypertrophy. Lysozyme, a protein similar in charge and molecular size to α -lactalbumin (Shewale *et al.*, 1984) had no effect on the bulk diet-induced gland hypertrophy. The introduction of the monospecific antibody (intraperitoneal injection) with the bulk-diet was able to inhibit gland enlargement, while the control pre-immune serum had no effect. While α -lactalbumin blocked parotid gland hypertrophy in bulk diet-fed animals, 4β -galactosyltransferase activity was induced to the levels observed for the bulk diet alone (Table 3). This finding was similar to that previously observed for α -lactalbumin inhibition of isoprenaline-mediated parotid gland hypertrophy (Humphreys-Beher, 1987). Induced proline-rich protein synthesis and 4β -galactosyltransferase activity were detected, although parotid gland hypertrophy did not take place in the presence of α -lactalbumin (results not shown; Table 3).

The marked increase in cell surface 4β -galactosyltransferase of acinar cells of parotid stimulated chronically by isoprenaline (Humphreys-Beher, 1987) may be related to activation of β -adrenergic receptors. The mechanism

Table 3. Inhibition of parotid gland hypertrophy by specific 4 β -galactosyltransferase substrates

Treatment	Gland weight* (mg)	[³ H]Thymidine incorporation† (c.p.m./mg of protein)	Total membrane 4 β -galactosyl- transferase activity‡
Chow diet	180 $\pm$ 90	225 $\pm$ 25	0.23 $\pm$ 0.08
Bulk diet	421 $\pm$ 17	1145 $\pm$ 43	1.14 $\pm$ 0.13
Bulk diet/10 mM-UDP-Gal	176 $\pm$ 27	305 $\pm$ 17	1.01 $\pm$ 0.10
Bulk diet/100 μ M-UDP-Gal	268 $\pm$ 22	721 $\pm$ 10	1.15 $\pm$ 0.20
Bulk diet/1 μ M-UDP-Gal	339 $\pm$ 37	975 $\pm$ 16	0.98 $\pm$ 0.17
Bulk diet/10 mM-GDP-Man	407 $\pm$ 25	1200 $\pm$ 50	1.00 $\pm$ 0.13
Bulk diet/10 mM-UDP-Glc	413 $\pm$ 13	1095 $\pm$ 20	1.11 $\pm$ 0.15
Bulk diet/10 mM- α -lactalbumin	182 $\pm$ 9	242 $\pm$ 15	1.16 $\pm$ 0.21
Bulk diet/100 μ M- α -lactalbumin	293 $\pm$ 14	312 $\pm$ 10	1.35 $\pm$ 0.25
Bulk diet/1 μ M- α -lactalbumin	364 $\pm$ 18	978 $\pm$ 12	1.21 $\pm$ 0.24
Bulk diet/10 mM-lysozyme	422 $\pm$ 21	1120 $\pm$ 27	1.08 $\pm$ 0.11
Bulk diet/pre-immune serum	445 $\pm$ 13	1090 $\pm$ 30	1.10 $\pm$ 0.27
Bulk diet/antiserum to 4 β -galactosyltransferase	179 $\pm$ 19	230 $\pm$ 15	1.16 $\pm$ 0.12

* Values determined for three experimental animals, expressed as mean $\pm$ S.D.

† Values determined from two experimental animals with assays performed in triplicate.

‡ Values determined from three experimental animals performed in triplicate. Enzyme activity is expressed as nmol/min per mg of membrane protein.

responsible for the apparent change in subcellular localization of the 4 β -galactosyltransferase remains unknown. The increase in mRNA levels for this enzyme after isoprenaline stimulation is the result, however, of an increase in transcription from a single gene (Humphreys-Beher *et al.*, 1986). It is now evident that not only excessive activation of β -adrenergic receptors, but also cholinergic receptors in neural stimulation caused by dietary changes, can cause changes in gene expression for the 4 β -galactosyltransferase. It is therefore of interest to note that the effects of the cholinergic pathway alone in mediating gene expression are now being detected.

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