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β -Adrenergic Activation of Glycosyltransferases in the Dolichylmonophosphate-Linked Pathway of Protein N-Glycosylation

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Summary. β-Adrenoreceptor stimulation of rat parotid acinar cells increases the activity of several microsmal membrane associated, dolichylmonophosphate (Dol-P) linked glycosyltransferases. The activities of Man-P-Dol synthase and Glc-P-Dol synthase are increased by ∿50%, and the activity of N-acetylglucosaminyl 1-phosphate transferase plus N-acetylglucosaminyl transferase increased by ∿60%, after agonist treatment. Increases in enzyme activity are (i) independent of endogenous Dol-P levels and (ii) observed under conditions in which the specific activities of donor sugar nucleotides are kept constant. Activation of these enzymes is specific since comparable levels of NADPH-cytochrome c reductase are found in control and agonist-treated membranes. The data thus provide the initial demonstration of neurotransmitter modulation of enzymes in the dolichol-linked pathway of protein N-glycosylation.

The biosynthesis of oligosaccharides and their attachment to protein asparagine residues is a multi-step process which has received considerable attention in the last decade (1). The process of oligosaccharide assembly on a dolichylmonophosphate carrier, as well as the "en bloc" transfer of the oligosaccharide chain to the appropriate protein-acceptor site, have been intensively studied (2-4). Although all of the enzymes involved in oligosaccharide-PP-dolichol synthesis (1,3) and the oligosaccharride transferase (5,6) have been well characterized, little is known about ways by which cells may regulate the activity of these enzymes.

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Abbreviations used: Dol-P, dolichylmonophosphate; Man-P-Dol, mannosylphosphoryl dolichol; Glc-P-Dol, glucosylphosphoryl dolichol; GlcNAc-PP-Dol, N-acetylpyrophosphoryl dolichol; $(GlcNAc)_2$ -PP-Dol, N,N'-diacetylchitobiosylpyrophosphoryl dolichol

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Several reports have suggested that extracellular signalling may contribute to the regulation of events leading to N-protein glycosylation (7-11). Earlier we demonstrated that concomitant with eliciting exocrine protein secretion, stimulation at the β-adrenoreceptor markedly enhances lipid-linked protein glycosylation in rat parotid gland acinar cells through a cyclic AMP mediated mechanism (9,12). We now report that microsomal membranes from rat parotid acinar cells, pretreated with the β-adrenergic agonist isoproterenol, enzymatically transfer N-acetylglucosamine 1-phosphate, N-acetylglucosamine, mannose and glucose, from their respective nucleotides to Dol-P at a much faster rate than seen with untreated membranes. The data support a role for neurotransmitter control of "key" glycosyltransferases in the process of N-linked protein glycosylation.

MATERIALS AND METHODS

Materials. Three month old male Wistar rats were purchased from Harlan Sprague Dawley. UDP-[U-^{14}C] glucose (233 mCi/mmol), UDP-N-acetyl [U-^{14}C] glucosamine (268 mCi/mmol) and GDP-[U-^{14}C] mannose (307 mCi/mmol) were purchased from Amersham Corp. Tunicamycin and dolichylmonophosphate were obtained from Calbiochem. Amphomycin (Ca^{2+} salt) was a gift from Bristol Laboratories and Dr. M. Bodanszky. All other chemicals were the highest grade commercially available and were acquired from previously reported sources (9,12).

Preparation of microsomal membrane enzyme source. Dispersed parotid acinar cells, prepared as described (9,12) were incubated at 37^{0}C in a shaking water bath in the presence and absence of 10^{-5}M isoproterenol in Ham's F-12 medium for 1 hr with gassing (95% O_2-5% CO_2) at 20 min intervals. At the end of incubation, cells were separated by brief centrifugation (15 sec at 40xg), resuspended in 0.1 M Tris-HCl, pH 7.0 containing 0.25 M sucrose, 1 mM EDTA and homogenized with a Brinkman Polytron (setting 5 for 10 sec). The microsomal membranes were prepared following a procedure described for calf brain (13) with a minor modification. Briefly, the homogenate was centrifuged at 600xg for 10 min and the resulting supernatant at 9000xg for 10 min. This supernatant was then centrifuged at 39,000xg for 15 min and the resulting particulate fraction suspended in the homogenization buffer and frozen in multiple aliquots until used as the microsomal enzyme source.

Assays of glycosyltransferases. The enzymatic transfer of N-acety-[^{14}C] glucosamine (13), [^{14}C] mannose (14) and [^{14}C] glucose (15) from their nucleotide derivatives to membrane-associated endogenous acceptors was assayed as described. Protein was measured by the Bio-Rad protein assay with bovine serum albumin as a standard. Radioactivity incorporated into various saccharide-lipids was assayed by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Enzymatic synthesis of GlcNAc-PP-Dol and $(GlcNAc)_2$-PP-Dol by N-acetylglucosaminyl 1-phosphate transferase and N-acetylglucosaminyl transferase respectively, are the initial steps in N-linked oligosaccharide assembly. The activity of each of these enzymes as well as their sensitivity to tunicamycin was examined. As shown in Table 1, microsomal membranes from isoproterenol-treated cells display markedly higher activity of both enzymes. Formation of the initial saccharide-lipid GlcNAc-PP-Dol, is increased ~ 50% while that of the subsequent product, $(GlcNAc)_2$-PP-Dol, is elevated ~ 200% compared to control levels. It should be emphasized that since GlcNAc-PP-Dol generated is utilized in the formation of $(GlcNAc)_2$-PP-Dol, the marked difference in the percent enhancement of activity between the two enzymes may be due to a high turnover rate of GlcNAc-PP-Dol. Total formation of GlcNAc-lipids (i.e. both enzyme activities) is increased ~ 60% in membranes from isoproterenol-treated cells.

Table 1

Effect of exogenously added dolichylmonophosphate and tunicamycin on the synthesis of GlcNAc-PP-Dol and $(GlcNAc)_2$-PP-Dol by membranes isolated from untreated-and isoproterenol (10^{-5}M)-treated parotid acinar cells

Additions	GlcNAc-PP-Dol (pmol/mg protein)	$(GlcNAc)_2$-PP-Dol (pmol/mg protein)
Control Membrane (CM) (n=9)	20.85 ± 2.20	9.98 ± 1.39
CM + Dol-P (50ug) (n=4)	21.87 ± 0.18	7.22 ± 1.24
CM + Tunicamycin (1.5ug) (n=3)	6.11 ± 1.26	5.25 ± 1.45
Isoproterenol treated Membrane (IM) (n=9)	25.38 ± 2.69	25.56 ± 1.74
IM + Dol-P (50ug) (n=4)	29.10 ± 0.10	17.53 ± 2.65
IM + Tunicamycin (1.5ug) (n=3)	9.50 ± 2.39	8.37 ± 2.96

GlcNAc-lipid synthesis was assayed by incubating membranes in 5mM Tris-HCl, pH 7.0, 12.5mM-sucrose, 50uM EDTA and 5uM UDP-N-acetyl-[U-^{14}C] glucosamine (531 cpm/pmol) and 5mM $MgCl_2$ in a total volume of 0.1 ml at 37^{0}C for 10 min. The isolated lipid was then subjected to SG-81 paper chromatography in chloroform-methanol-H_2O (75:25:4). The separated lipids were then counted. Dol-P was dispersed in 10% DMSO and the final concentration of DMSO in each incubation was 0.5%. The data presented above are mean ± SEM from the number of experiments indicated in the parentheses.

Importantly when exogenous dolichylmonophosphate was added to the incubation mixtures, enzyme activities in membranes from agonist-treated cells remained markedly above that of controls. Such results suggest that levels of dolichylmonophosphate are not rate limiting for ß-adrenoreceptor activation of these glycosyltransferases. Similar findings were also observed with other dolichylmonophosphate-linked enzymes studied here (see below).

The formation of GlcNAc-lipids, in isoproterenol stimulated microsomal membranes, should be sensitive to treatment with tunicamycin, an antibiotic which specifically inhibits N-acetylglucosaminyl 1-phosphate transferase (16). As shown in Table 1, tunicamycin markedly inhibited GlcNAc-PP-Dol synthesis and as a result also $(GlcNAc)_2$-PP-Dol formation, in both control and agonist-treated membranes.

The four outer mannose residues found in the oligosaccharide-PP-Dol (Man_9 $GlcNAc_2$-PP-Dol) are added to the growing oligosaccharide from Man-P-Dol. The formation of this mannolipid is catalyzed by the enzyme Man-P-Dol synthase. As shown in Table 2 activity of the enzyme is increased considerably in membranes from isoproterenol-treated cells. Transfer of [^{14}C] mannose from GDP [^{14}C] mannose into Man-P-Dol is enhanced ~ 40% in the absence, and ~ 45% in the presence, of exogenous dolichylmonophosphate.

Table 2

Effect of exogenous dolichylmonophosphate on the transfer of mannose from GDP-mannose into parotid gland acinar cell membranes

	Man-P-Dol (pmol/mg protein)	
Additions	Control	Isoproterenol-treated
None (n=10)	89.98 ± 10.57	124.31 ± 14.71
+ Dol-P (50ug) (n=4)	153.96 ± 60.91	223.79 ± 54.09

Mannolipid synthesis was assayed by incubating membranes in 5 mM Tris-HCl, pH 7.0, 12.5 mM sucrose, 50uM EDTA, 2.5uM GDP-[U-^{14}C]-mannose (260 cpm/pmol) and 5mM $MnCl_2$ in a total volume of 0.1 ml at 37°C for 5 min. The amount of mannolipid synthesized was assayed and the data presented above are mean ± SEM of the number of experiments indicated in parentheses. Dol-P was dispersed in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in each incubation was 0.5%.

Table 3

Effect of exogenous dolichylmonophosphate and amphomycin on the transfer of glucose from UDP-glucose into parotid gland acinar cell membranes

	Glc-P-Dol (pmol/mg protein)	
Addition	Control	Isoproterenol-treated
None	108.26 ± 6.42	148.86 ± 5.23
+ Dol-P (50ug)	148.67 ± 5.02	185.31 ± 3.90
+ Amphomycin (5ug)	26.64 ± 1.16	36.53 ± 1.20

Glc-P-Dol synthesis was assayed by incubating membranes in 5mM Tris-HCl, pH 7.0, 12.5 mM sucrose, 50uM EDTA, 5uM UDP-[U-^{14}C] glucose (473 cpm/pmol) and 5mM $MgCl_2$ in a total volume of 0.1 ml at 37^{0}C for 10 min. The isolated lipid was subjected to SG-81 paper chromatography in chloroform-methanol-H_2O (60:25:4). The spots corresponding to Glc-P-Dol were counted. The data presented above are mean ± SEM from 5 separate determinations. Dol-P was dispersed in 10% DMSO and the final concentration of DMSO in each incubation was 0.5%.

Transfer of glucose from Glc-P-Dol to the growing oligosaccharide is considered to be the final step required for its "en bloc" transfer to the nascent polypeptide chain. As shown in Table 3, the level of Glc-P-Dol synthesis, i.e. activity of Glc-P-Dol synthase, is substantially higher in isoproterenol-treated membranes (~ 40%). Exogenously added dolichylmonophosphate considerably enhances Glc-P-Dol synthesis in both membrane preparations; agonist-treated membranes still showing much greater enzyme activity than controls (~ 25%). Furthermore, the lipopeptide antibiotic, amphomycin, which inhibits the formation of monosaccharide lipids involving dolichylmonophosphate by interacting specifically with Dol-P (4), profoundly inhibited (~75%) Glc-P-Dol synthesis comparably in both types of parotid membranes. The comparable inhibition of Glc-P-Dol synthesis by amphomycin in both types of parotid membranes also supports the idea that enhanced monosaccharide lipid synthesis is due to increases in enzyme activity rather than increase in endogenous dolichylmonophosphate.

Our earlier studies (9,12) with intact cells had indicated that enhanced mannose incorporation into parotid secretory glycoproteins was reflective of increased N-linked glycosylation because it was (a) blocked by tunicamycin and

(b) all newly incorporated mannose was found in mild base resistant glycopeptides. The results of the present studies lend convincing support for this conclusion by showing, in vitro, with microsomal membranes that tunicamycin blocks isoproterenol-stimulated formation of GlcNAc-PP-Dol and that amphomycin inhibits isoproterenol-stimulated synthesis of Glc-P-Dol.

In the present studies microsomal membranes were incubated at a saturating concentration of sugar nucleotides with constant specific activity. Also 2.5 mM 5'-AMP was included to limit nucleotidase activity and gave the same results (not shown). In addition when exogenous Dol-P was added to incubations, all enzymes were consistently more active in isoproterenol-treated membranes. Finally, the effects observed were not due to a non-specific activation of microsomal membrane-associated enzymes. Measurement of NADPH-cyctochrome c reductase (17), a marker for endoplasmic reticulum, indicated no differences in control (0.0062 umol cytochrome c reduced/min·mg protein) and isoproterenol-treated (0.0054 umol cytochrome c reduced/min·mg protein) membranes. Our results are indeed consistent with and provide the first demonstration of, neurotransmitter activation of "key" enzymes in the dolichol-linked cascade of glycosylation.

Since isoproterenol activates cyclic AMP-dependent protein kinases, (18-20) it is tempting to suggest that the enhancement of glycosyltransferase activities seen here may in part be due to cyclic AMP-dependent protein kinase mediated phosphorylation of these enzymes.

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