Uptake of dolichol into cultured cells

G. Palamarczyk* and T.D. Butters

National Institute for Medical Research, Mill Hill, London NW7 1AA, England

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Dolichol uptake *Glycosylation* *Sub-cellular fractionation*

1. INTRODUCTION

Dolichol is one of a series of polyprenoids that act as intermediates in glycosylation reactions [1]. In mammalian tissues dolichol-linked glycosylation is involved in the assembly of *N*-acetylglucosamine –asparagine-type chains or N-glycans [2]. Several drugs are available that inhibit partially or completely various stages of *N*-glycan assembly. Tunicamycin blocks the first step in the formation of the glycosylated lipid carrier, namely the production of dolichol diphosphate *N*-acetylglucosamine from dolichol phosphate and UDP-*N*-acetylglucosamine [3]. Amphomycin [4], showdomycin [5] and 2-deoxyglucose [6] inhibit formation of dolichol phosphomannose and dolichol phosphoglucose which are inportant intermediates for the transfer of mannose and glucose to a dolichyl oligosaccharide that is the immediate precursor for *N*-glycosylation of proteins. These drugs have been used to study the biological roles of glycoproteins carrying *N*-glycans. Tunicamycin treatment of cultured cells, for example, induces a significant reduction in the adhesiveness of cells to substrata [7] and inhibits the normal development of embryonic tissues [8,9]. To date, however, little attention has been given to the effects on cells of inducing a positive rather than an inhibitory modulation on protein glycosylation. Dolichol and dolichol phosphate are present in most cells at levels likely to be rate-limiting given the average rates of nascent protein synthesis in the endoplasmic reticulum [10,11]. We have considered the possibility therefore of accelerating the rate of protein glycosylation by providing cells with a source of exogenous dolichol, a lipophilic component that would be expected to by-pass the plasma membrane. We have surveyed a variety of cell types for their ability to take up dolichol from the external medium. The site of sequestration of dolichol in one cell type, mosquito larval cells, has been determined and the further metabolism of the cell-bound dolichol has been examined.

* Permanent address: Institute of Biochemistry and Biophysics, Warsaw, Poland

Address correspondence to T.D.B.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Dolichol from *Trichosantes palmata* was tritiated as in [12]. Silica gel 60 plates (20 × 20 cm) precoated, were obtained from E. Merck (Darmstadt). Pure chromatography solvents and NP40 were obtained from BDH (Poole, Dorset). [^{14}C]Mannose (83 mCi/mmol), D-[U-^{14}C]sorbitol (333 mCi/mmol) were from Amersham International (Bucks). Non-radioactive sugar nucleotides, ATP, CTP, AMP, cholesterol and dolichol phosphate were from Sigma Chemicals (St Louis MO).

2.2. *Cells*

Baby hamster kidney cells (BHK C13) were grown in monolayer culture in modified Eagles medium supplemented with 10% foetal calf serum and antibiotics (BHK medium) at 38°C. Mosquito larval cell line Mos 20A was grown at 28°C in medium [13]. Primary chick embryo fibroblasts were provided by Dr A. Hay (NIMR) and grown in the BHK medium at 38°C. *Xenopus* hepatocyte cultures were provided by Dr C. Watkins (NIMR).

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Drosophila cell lines were obtained from Dr A. Flavell (Imperial Cancer Research Fund Laboratories, Mill Hill).

2.3. *Uptake experiments*

Cells growing in serum-supplemented medium as monolayers in Flow multi-well culture vessels (2 cm^2 well area) were washed once with serum-free mosquito cell culture medium and incubated at the appropriate temperature with 0.5 ml [^{3}H]dolichol (6×10^5 cpmspec.act.4×10^9 cpm/μmol).Portions of the radioactive dolichol in benzene were evaporated under nitrogen and resuspended in serum-free medium containing 0.5% DMSO. After incubation the cells were washed 3 times with phosphate-buffered saline (P_i/NaCl), 0.5% DMSO (1 ml each time), scraped from the culture wells with a rubber policeman and pelleted by centrifugation. The cells were then extracted twice with 2 ml each of chloroform:methanol (3:2, v/v) at room temperature for 10 min. The combined supernatants obtained after centrifugation were partitioned with 4 mM $MgCl_2$, the upper phase removed and the lower phase washed twice with theoretical Folch upper phase solvent. Aliquots of washed, lower phase extracts were taken for radioactivity measurements and for thin-layer chromatography. The residues were dissolved in 0.5 N NaOH (0.5 ml) and analysed for protein [7]. Results are expressed as uptake of [^{3}H]dolichol/mg cell protein. Uptake of [^{14}C]-sorbitol was measured by thorough washing of the monolayer cultures with P_i/NaCl followed by solubilization of the cells with alkali (1 ml). Aliquots were then taken for estimation of protein and for radioactive counting using a silica-based scintillation fluid [7].

2.4. *Sub-cellular fractionation*

Mosquito 20A cells ($\sim 10^9$) were mixed with cells ($\sim 10^8$) that had been cultured at 28°C overnight with [^{3}H]dolichol ($5-8 \times 10^6$ cpm). The cells were harvested, washed as before, suspended in 10 mM Tris–HCl (pH 7.4) (40 ml) and broken by Dounce homogenisation. The crude nuclear fraction and unbroken cells were removed by centrifugation at $1000 \times g$ and the supernatant centrifuged through a discontinuous sucrose gradient (20–50%) as in [13]. Protein material at sucrose interfaces was recovered by centrifugation (100 000 × *g*, 1 h, 2°C) and purified further by ultracentrifugation through the same discontinuous sucrose gradient. The protein material in the 20% sucrose layer ($A1_L$), at the 20–30% ($A1_H$, A2) and 30–40% (A3) interfaces and at the bottom of the gradient (A4) were recovered as above and finally purified by centrifugation through continuous sucrose gradients [13]. Material sedimenting in the 40–50% sucrose layers from the first centrifugation was also recentrifuged on a discontinuous sucrose gradient [13] to separate 2 protein bands visible in the 40% sucrose layer (B1, B2) and a band at the 40–50% sucrose interface (B3). These were also centrifuged through continuous sucrose gradients as a final purification step [13]. The assignment of subcellular location for each fraction was determined by assays of appropriate enzyme markers as in [13]: $A1_L$ and $A1_H$, plasma membranes; A2 and A3, smooth internal membrane; A4 and B1, rough endoplasmic reticulum; B2 and B3 mitochondrial enriched fractions.

Nuclear sub-fractions were also obtained labelled cells lysed with 10 mM Tris–HCl (pH 7.4) containing 5 mM $MgCl_2$. The published procedure [14] was followed and the fractions (N1–N6 and pellet) obtained. Fractions N2–N6 contained sufficent material for further analysis.

2.5. *Identification of products*

Chloroform:methanol (3:2, v/v) extracts of labelled cells were spotted onto silica gel plates and analysed by ascending chromatography in chloroform:methanol:water (65:25:4, by vol.) or ethylacetate:benzene (5:95, v/v). Chromatography was performed until the solvent front had reached 2 cm from the top of the plates. Chromatograms were sprayed with chloroform containing 10% PPO, dried and set up for radio-fluorography with Fuji RX film or tritium sensitive Ultrofilm (LKB). In some cases the silica gel was scraped from the appropriate areas of the plates and material eluted with chromatography solvent for measurement of radioactivity.

DEAE Sephadex A-50 chromatography of lipid extracts was performed using 4 × 0.5 cm columns. Elution was with 10 ml aliquots of chloroform:methanol:water (1:1:0.3, by vol.) followed sequentially by 10 mM, 50 mM and 100 mM ammonium acetate in methanol. The various fractions were concentrated by drying under nitrogen and spotted onto silica gel plates as described above.

2.6. *Estimations of enzyme activities in mosquito cells*

The amounts of dolichol phosphomannose synthetase was meassured as in [13] using exogenously added C_{100} dolichol phosphate. Product conversion of the [^{3}H]dolichol incorporated into cell fractions was determined by incubating aliquots of sub-cellular fractions (100 μg protein) with Tris–HCl (pH 7.4) (11 mM), EDTA (0.3 mM), β-mercaptoethanol (5.5 mM), $CaCl_2$ (60 mM), $MgCl_2$ (11 mM), NP40 (0.26%, v/v), AMP (2.7 mM), CTP (16.5 mM), GDP-mannose (0.54 mM) and UDP-*N*-acetylglucosamine (0.54 mM) for 40 min at 30°C. Lipid material was extracted with chloroform: methanol (3:2, v/v) and loaded onto thin-layer silica gel plates for chromatography using chloroform:methanol:water (65:25:4, by vol.). Dry plates were treated for radio-fluorography as described.

3. RESULTS

3.1. *Incorporation of dolichol*

Dolichol was rapidly taken up by mosquito cells from the external medium (fig.1A). The absolute amounts taken up over 24 h varied from 35–75% of the total dolichol added. Cells cultured at low density appeared to take up rather more dolichol than cells at higher density and for subsequent experiments confluent cultures were used. Temperature had little effect: cells incubated at optimum temperature for growth (28°C) incorporated dolichol at similar initial rates as cells kept at 36°C. Serum had an inhibitory effect on dolichol uptake after 8 h incubation, perhaps due to binding to serum lipoproteins (fig.1A).

Each of the cell lines tested also incorporated dolichol from the culture medium but to significantly different extents (fig.1B–D). The initial rates of incorporation (pmol dolichol . min^{-1} . 10^6 cells^{-1}) were as follows: chick embryo fibroblasts, 167; mosquito cells, 21.5; *Drosophila* cells, line ox, 64.3; line v, 27.8; BHK cells, 15.3; *Xenopus* hepatocytes, 0.9. In all cell lines incorporation was time dependent. The possibility that dolichol was being taken up by bulk phase endocytosis or other non-specific mechanisms was examined by comparing rates of incorporation of dolichol with a non-metabolizable label, [^{14}C]sorbitol. Sorbitol was taken up poorly and in a time-independent manner (fig.1A).

Insects depend on a dietary source of cholesterol since they are unable to synthesize the steroid ring [15]. Therefore, these cells might be expected to have highly efficient mechanisms for the transport of polyprenoid compounds across the cell membrane. However, addition of excess amounts of cholesterol and α-ecdysone, an insect steroid hormone, to dolichol containing medium were found to have no inhibitory effect on the uptake of dolichol by mosquito cells (table 1) indicating independent uptake mechanisms.

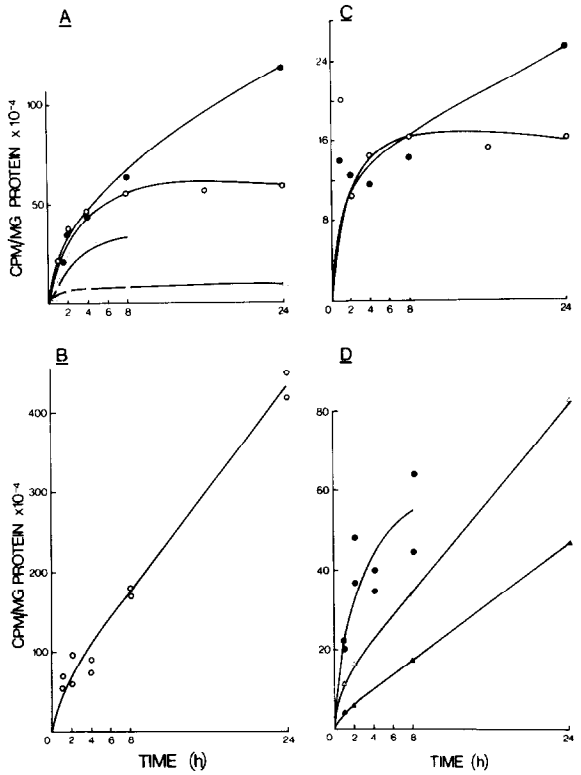

Fig.1. Uptake of [^{3}H]dolichol by cultured cells. Multiwell culture vessels were incubated with 6 × 10^5 cpm of radioactively labelled dolichol for the times shown and the incorporation into protein determined as in section 2. (A) Mosquito cells: (○——○) at 28°C; (●——●) at 36°C; (△——△) at 28°C in the presence of 15% foetal calf serum; (□——□) at 28°C with [^{14}C]sorbitol, 6 × 10^5 cpm instead of labelled dolichol. (B) Chick embryo fibroblasts at 36°C. (C) BHK cells (○——○) at 28°C; (●——●) at 36°C. (D) *Xenopus* cells (●——●) at 36°C; *Drosophila* cells (△——△) at 28°C, ox. line; (▲——▲) at 28°C, v line.

Table 1

Uptake of [^{3}H]dolichol by mosquito cells in the presence of cholesterol and ecdysone

No.	Additions		pmol/mg protein
1.	None		149–219
2.	Cholesterol	(25 μg) 65 nmol	173
3.	Cholesterol	(50 μg) 130 nmol	151
4.	Ecdysone	(25 μg) 50 nmol	209
5.	Ecydsone	(50 μg) 100 nmol	214

Mosquito cells growing as monolayers in multi-well dishes were labelled with 6×10^5 cpm (150 pmol) of [^{3}H]dolichol in medium containing the additions as shown. After 8 h at 28°C the amount of radioactivity incorporated was measured as described in the text

3.2. *Sub-cellular localisation of incorporated dolichol*

We have described a scheme of sub-cellular fractionation of cultured mosquito cells [13]. Fractions enriched in plasma membranes, smooth internal membranes, rough endoplasmic reticulum, mitochondria and nuclear material were characterised. Table 2 summarises the data obtained for the sub-cellular location of exogenous dolichol taken up by cells under conditions shown in fig.1A. The highest specific activity of [^{3}H]dolichol incorporated mg protein was found associated with fractions enriched in plasma membranes $A1_L$ and $A1_H$. During subsequent centrifugation of these fractions through continuous sucrose density gradients, [^{3}H]-dolichol radioactivity and membrane material sedimented co-incidently (not shown). Other internal

Table 2

Fractionation of [^{3}H]dolichol-labelled mosquito cells and levels of dolichol phosphomannose synthetase

	^{3}H-radioactivity		Synthetase activity	
	Total cpm $\times 10^{-5}$	Spec. act. (cpm/mg protein) $\times 10^{-4}$	Total (pmol/h) $\times 10^{-3}$	Spec. act. (pmol . h^{-1} . mg protein^{-1})
Exp. A				
Homogenate	65.40	2.39		
1000 × *g* pellet	4.20	4.80		
1000 × *g* supernatant	64.80	3.66		
Fraction $A1_L$	0.55	17.49	0.001	4.86
$A1_H$	2.52	13.36	0.348	184.44
A2	2.79	9.93	0.080	28.54
A3	0.92	5.59	0.019	11.83
A4	1.18	5.19	0.399	176.78
B1	1.64	2.86	13.830	2409.41
B2	0.50	1.19	13.590	3228.03
B3	0.93	1.78	10.960	2087.62
Exp. B				
1000 × *g* pellet	6.90	3.82		
Fraction N2	1.41	7.88	0.176	98.50
N3	0.27	1.96	0.004	3.40
N4	2.89	4.91	0.027	4.73
N5	0.09	4.86	0.0	0.0

Mosquito cells were labelled overnight with [^{3}H]dolichol (1.2×10^6 cpm) and subcellular fractions obtained as described in the text. Portions of purified fractions were assayed for synthetase activity using [^{14}C]GDP-mannose as described

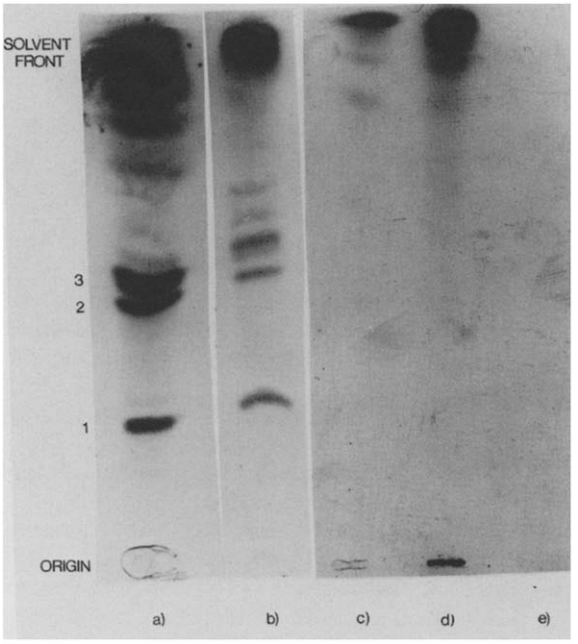

Fig.2. Chromatography of radioactive components extracted from mosquito cells after uptake of [^{3}H]dolichol. Cell extracts (a,b) or an equivalent amount of [^{3}H]dolichol (c–e) were eluted from a DEAE–Sephadex column with chloroform:methanol (3:2, v/v) (a,d) followed by 50 mM ammonium acetate (b,e). Eluates were concentrated and loaded onto TLC plates and the subsequent chromatogram developed for radioactivity. [^{3}H]Dolichol before ion-exchange chromatography is shown in (C).

membrane (A2, A3, A4, B1) and mitochondrial fractions (B2, B3) also contained high amounts of labelled dolichol but of a much lower specific activity. It was noted that the 1000 × *g* pellet containing nuclei and a few unbroken cells incorporated relatively little exogenous dolichol (4.2 × 10^5 cpm) compared to the 1000 × *g* supernatant but at a higher specific activity. Further examination of this fraction (exp. B, table 2) showed that subfractions of nuclear membranes (fraction, N2 and N4) were enriched for the dolichol label.

Estimations of the levels of dolichol phosphomannose synthetase activity (table 2) identified the heavier sucrose banding fractions, B1, B2 and B3 as being markedly enriched in enzyme activity. These fractions contained large amounts of rough endoplasmic reticulum and mitochondrial marker enzyme activities. Although some synthetase activity was associated with smooth membrane fractions (especially $A1_h$) and nuclear fraction N2, there appeared little correlation between the site of dolichol glycosylation and incorporation of exogenous dolichol into sub-cellular fractions.

3.3. *Identification of [^{3}H]dolichol-labelled products in mosquito cells*

When mosquito cells were incubated with [^{3}H]-dolichol and chloroform:methanol extracts of labelled material separated by ion-exchange chromatography, thin-layer chromatograms detected slow-moving products in addition to free dolichol (fig.2a,b). In both chloroform:methanol:water and 50 mM ammonium acetate eluates 3 bands were prominent (bands 1, R_F 0.29; 2, R_F 0.50; 3, R_F 0.56), suggesting that labelled dolichol is converted to the phosphorylated form (band 3) which then undergoes substitution with carbohydrate residues to give slower moving bands 1 and 2. Authentic samples of dolichol phosphate and dolichol phosphomannose migrated with identical R_F-values to bands 3 and 2, respectively. Band 1 corresponded to the expected mobility of dolichol diphosphate *N*-acetylglucosamine in the TLC system used [16]. An equivalent amount of [^{3}H]dolichol used to label cells was taken and subjected to chromatography (fig.2c–e). No slower moving products corresponding to bands 1–3 were observed.

4. DISCUSSION

Exogenously added dolichol is transferred into cells where it is associated with intra-cellular membranes and organelles [17]. Here we have shown that in a cultured mosquito cell line the majority of the dolichol which is taken up by cells remains bound to the plasma membrane-enriched fractions. A small but detectable amount of the incorporated labelled dolichol is modified by phosphorylation and by substitution with carbohydrate groups, reactions which are generally ascribed to the rough endoplasmic reticulum of cells [2]. In agreement with this, we found that the major site for synthesis of dolichol phosphomannose in mosquito cells to be those sub-cellular fractions enriched in rough endoplasmic reticulum membranes. These fractions are also rich in mitochondrial membranes which have been found to be involved in the synthesis of dolichol from mevalonic acid [18] and its conver-

sion to glycosylated forms [19,20]. Better separation techniques will be required to determine the precise location of similar enzymes in mosquito cells.

We have examined a number of cell lines from different species and genera and estimated the extent to which lipid carriers are taken up. Some cells are more efficient than others in uptake, and given that compositional differences exist between plasma membranes from different genera [13], the presence or activation of specific membrane transport mechanisms could determine relative uptake efficiences. The addition of dolichols to Erlich ascites tumour cells has been reported to induce attachment and spreading to glass substrata [21], events in which cell surface carbohydrates are implicated [22]. Modifications of cytoplasmic glycosylation reactions by cellular uptake of dolichol may be useful in determining the exact roles of cell surface glycocojugates in such biologically important processes.

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