Glycoprotein Synthesis in Drosophila Kc Cells

BIOSYNTHESIS OF DOLICHOL-LINKED SACCHARIDES*

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The biosynthesis of dolichol and dolichol-linked saccharide intermediates in glycoprotein synthesis was studied in an embryonic Drosophila cell line (Kc) that lacks the squaene-cholesterol branch of the polyisoprenoid biosynthetic pathway. Kc cells were labeled with [5-3H]mevalonic acid and the radioactive lipids formed were analyzed. Although the major labeled product was coenzyme Q, dolichol and a variety of dolichol derivatives could be readily detected. On the basis of their chromatographic and chemical properties, these derivatives were identified as dolichyl phosphate, glucosylphosphoryldolichol, mannosylphosphoryldolichol, and oligosaccharylphosphoryldolichol. Both short term (4-h) and steady state (4-day) labeling experiments with mevalonate, rather than sugars as previously used, were performed to assess the level of these intermediates. The results of these studies, using a precursor common to all the intermediates, reveal that the early intermediates, N-acetylglucosaminylphosphoryldolichol and N,N'-diacetylichitobiosylphosphoryldolichol, are present at very low levels (<5%) relative to the other intermediates on the pathway to oligosaccharylphosphoryldolichol. The total amount of dolichol intermediates remained essentially constant during the chase phase of pulse-chase experiments, indicating the absence of a major catabolic pathway mediating the polyisoprenoid backbone. As expected, however, the sugar moiety, studied with mannosylphosphoryldolichol, underwent rapid turnover. These results are discussed in the context of our current understanding of the pathway whereby dolichol derivatives participate in glycoprotein synthesis.

Among higher organisms, insects are unique because they cannot synthesize cholesterol, a result of the absence of the enzyme that converts farnesylpyrophosphate to squaene. N-linked polysaccharide glycoproteins have been shown to be synthesized in a mosquito cell line (1), and the detection of sugar-linked dolichol derivatives (2) indicates that the glycoprotein assembly process in insects is analogous to that found in higher organisms. Given these facts and the extensive studies by Watson and co-workers (3-6) that showed that Drosophila Kc cells utilize the mevalonate pathway for synthesis of dolichol and coenzyme Q, it appeared that these cells would be ideal to study the synthesis and metabolism of dolichol and its glycosylated derivatives that are involved in glycoprotein synthesis.

Numerous studies (reviewed in Ref. 7) have examined the synthesis and metabolism of these derivatives based on sugar-labeling experiments rather than on measurements of the polyisoprenoid chain to which the saccharides are attached. It has been necessary to use sugars rather than mevalonate for labeling these compounds, because in most higher organisms dolichol and its compounds share a common biosynthetic pathway with cholesterol, which often exceeds their amounts by 100-1000-fold. Consequently, it was expected that in Drosophila cells the difficulty of isolation and quantification of the various glycosylated derivatives containing label in the dolichol chain would be obviated.

The results of these studies revealed that the major mevalonate-derived lipid product formed in Drosophila Kc cells and extractable by CHCl3:CH3OH (2:1) was coenzyme Q. The dolichol-related compounds in this fraction were found to be free dolichol, dolichyl phosphate, mannosylphosphoryldolichol, and glucosylphosphoryldolichol. Little or no N,N'-diacetylichitobiosylphosphoryldolichol could be detected. Oligosaccharylphosphoryldolichol was identified in the CHCl3:CH3OH:H2O (10:10:3) extract from mevalonate-labeled cells. Pulse-chase experiments suggested that dolichyl phosphate is the initial product and that dolichol is slowly formed by its hydrolysis. The relative level of dolichol-linked saccharide derivatives was determined in short and long term labeling experiments. In addition, the effect of several agents on the synthesis of dolichol and its derivatives were studied. The results are discussed in the context of the relative levels and the dynamics of dolichol-linked intermediates in glycoprotein synthesis in these cells.

EXPERIMENTAL PROCEDURES

Materials—Coenzyme Q10, dolichol (grade I), dolichyl phosphate (grade II), and wheat germ acid phosphatase were products of Sigma. Octyl-β-D-glucoside, 20-hydroxyecdysone, and tunicamycin were purchased from Behring Diagnostics. (R)-[5-3H]Mevalonic acid triethylammonium salt (27.6 Ci/mmol), D-[2-3H]mannose (15 Ci/mmol), and D-[6-3H]glucosamine (25 Ci/mmol) were obtained from Du Pont-New England Nuclear. [1-3H]Dolichol (50 mCi/mmol) was kindly provided by Kurarai Co. (Kurarashi, Japan), and [1-14C]dolichyl phosphate was chemically synthesized from [1-14C]dolichol by using POCl3(8,9).[3H]Mannosylphosphoryldolichol, [3H]glucosylphosphoryldolichol, N-acetyl[3H]glucosaminylphosphoryldolichol, and N,N'-diacetyl[3H]chitobiosylphosphoryldolichol were synthesized with oviduct microsomes according to published methods (10) and were gifts from Dr. Howard A. Kaplan of this laboratory. All other chemicals were reagent grade.

Kc Cell Cultures—Kc cell cultures were generously provided by Dr. John A. Watson, Department of Biochemistry and Biophysics, University of California, San Francisco. Monolayer or suspension cultures of Kc cells were grown at room temperature in Echelier's D22 medium (11) with 1% fetal calf serum. The cultures were refed by

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dilution with fresh Echalar's D22 medium containing 1% fetal calf serum every 3-4 days. The cells grew in monolayer culture with a doubling time of 24 h. After transfer to suspension culture in stirring bottles, a lag was observed. After the lag (4 h), the doubling time was 32 h. In the cultures in which metabolic labeling was carried out, the viability of the cells was 90-95%.

Thin-layer Chromatography—Thin-layer chromatography (TLC) was carried out on Silica Gel G precoated glass plates or plastic plates (Merck) or on reversed-phase RP-18 precoated glass plates (Merck). The solvent systems used for development were as follows: solvent system A, petroleum ether:benzene:methanol (50:48:2); solvent system B, chloroform:methanol:concentrated ammonia:water (65:35:4:4); solvent system C, propyanaminewater (6:3:1); solvent system D, diisobutylketone:glacial acetic acid:water (60:45:6); solvent system E, benzene-ethyl acetate (4:1); solvent system F, acetoacetic acid; solvent system G, chloroform:methanol:water (10:10:3). The position of migration of authentic standards was visualized with iodine vapor. Radioactivity was determined on air-dried plates by scraping (glass plates) or cutting (plastic plates) 0.5 × 2-cm sections, which were then placed in scintillation vials containing 5 ml of Liquiscint (National Diagnostics, Somerville, NJ). For autoradiography, the plate was sprayed three times with ENHANCE and exposed in X-ray film for at least 30 h at −80°C for a week. The relative intensities of the bands of 3H- or 14C-labeled compounds were measured on film with a scanning densitometer.

For further analysis of the putative labeled dolichol, dolichyl phosphate, and glycosylphosphoryldolichol, preparative TLC was carried out and these compounds were recovered from the scraped silica gel with CHCl3:MeOH (10:1) (for component I) and CHCl3:MeOH:con- centrated NH4OH:H2O (65:35:4:4) (for components IV and V, see Fig. 1). Incorporation of [3H]Mevalonic Acid into Lipids—Specific experimental conditions are presented in the appropriate figure or table. The general method for labeling was as follows: Drosophila Kc cells (107-108 cells/ml) in suspension were harvested by centrifugation and the cell pellets were washed once with Echalar's D22 medium. The washed cells were suspended in fresh Echalar's medium containing 1% fetal calf serum. The cells were labeled with [3H]mevalonic acid at room temperature with stirring at 100 rpm and bubbled for 1 min every 20 min with air through a syringe fitted with a sterile filter. After being labeled for 4 h, the suspension was chilled on ice, centrifuged, and washed with ice-cold Robb's physiological saline (12). CHCl3:CH2OH (2:1) was added to the cell pellets, and the suspension was incubated at 37°C for 10 min. The extraction with CHCl3:CH2OH was repeated three times. The combined CHCl3:CH2OH extracts were back-washed once with 0.9% NaCl and then twice with 0.9% NaCl:CH3OH (1:1). The chloroform extracts were dried with water at least five times and then further extracted with CHCl3:CH2OH:H2O (10:10:3) three times.

For analysis, an aliquot of the CHCl3:CH2OH extract was subjected to ion-exchange chromatography on a DEAE-cellulose column equilibrated with CHCl3:CH2OH. After the column was washed with the same solvent, radioactive metabolites were eluted stepwise by 50, 100, and 200 mM CH3COONa in CHCl3:CH2OH. The uncharged fraction that did not bind to the DEAE-cellulose column and the charged fraction that was eluted by 50 mM CH3COONa in CHCl3:CH2OH were further analyzed by silica gel TLC.

Incorporation of [3H]Mannose and [3H]Glucosamine into Lipids and Proteins—Kc cells (2-3 × 107 cells/ml) in glucose-free Echalar's D22 medium containing 1% fetal calf serum were labeled with [3H]mannose (5 μCi/ml) or [3H]glucosamine (5 μCi/ml) at room temperature with stirring at 100 rpm. After being labeled for 4 h, the cells were chilled on ice and washed several times with glucose-free Echalar's medium containing cold mannose or glucosamine (20 mM) until no radioactivity in the supernatant was detected. The washed cell pellets were analyzed for protein- or dolichol-linked oligosaccharides.

To measure protein-linked oligosaccharides, 10% (v/v) of trichloroacetic acid was added to the pellets. The mixture was incubated at 100°C for 10 min, chilled, and stored. The precipitates were collected by centrifugation, washed two times with cold 10% trichloroacetic acid, and then washed four times with diethyl ether. The precipitates were dried with a stream of N2 and then digested with Pronase for 48 h as previously described (13). The isolated material was removed by centrifugation and the supernatant was analyzed by gel filtration on a Sephadex G-50 column equilibrated with 50 mM HCOONa. The radioactive fraction with V/Vc of 1.8 was collected and further analyzed by affinity chromatography on a concanavalin A-agarose column.

For determination of dolichol-linked saccharides, CHCl3:CH2OH was added to the cell pellet. The extraction was repeated three times and the combined extracts were back-washed with 0.9% NaCl and with 0.9% NaCl:CH3OH (1:1) twice and analyzed by ion-exchange chromatography on a DEAE-cellulose column equilibrated with CHCl3:CH2OH. The fractions eluted directly by 0.1 M CH3CONa in CHCl3:CH2OH were pooled and analyzed by silica gel TLC in solvent system B. The pellets insoluble in CHCl3:CH2OH were dried with a stream of N2 and washed with water at least five times until no radioactivity in the supernatant was detected. Then the wet pellet was dried with N2 and extracted with CHCl3:CH2OH:H2O three times in order to recover the oligosaccharide-lipid.

RESULTS

Characterization of Metabolites Extractable with CHCl3:CH2OH from Kc Cells Labeled with [3H]Mevalonic Acid

Kc cells were labeled with [3H]mevalonic acid and the resulting radioactive metabolites extractable with CHCl3:CH2OH were analyzed by the procedures described under "Experimental Procedures." When an aliquot of the CHCl3:CH2OH extract was analyzed by DEAE-cellulose chromatography, two peaks of radioactivity were observed (Fig. 1A). The fraction containing uncharged components not bound to DEAE-cellulose was analyzed by TLC. The major radioactive component (I) was found to correspond to coenzyme Q (Fig. 1B), as previously reported (3, 4). A second uncharged radioactive component (II), migrating slower than coenzyme Q, had the mobility of dolichol, whereas a third one coincided with authentic farnesol. Two other minor components, one with a mobility between that of components I and II and another remaining at the origin, were not identified.

The charged fraction, containing components bound to DEAE-cellulose, was analyzed by TLC. Two radioactivity peaks were observed; the slower moving one (V) migrated with the mobility of dolichyl phosphate (Fig. 1C) and the faster one (IV) with the mobility of glycosylphosphoryldolichol.

Further analysis was performed as follows on each of the following compounds after their isolation by preparative TLC as described under "Experimental Procedures."

Dolichol—The putative labeled dolichol fraction (II) co-chromatographed with authentic dolichol by TLC in solvent system E. Furthermore, it could be resolved into a family of dolichol homologues by reversed-phase TLC (Fig. 2). In contrast to the profile of pig liver dolichol, the insect dolichol consists of three homologues with the major carbon chain length ranging from C25 to C35. To test the possibility that the insect dolichol was actually unsaturated, i.e. didehydrodolichol, it and authentic 14C-labeled dolichol were separately treated with mild acid (14, 15) and analyzed by TLC in solvent system E. Neither authentic dolichol nor the insect dolichol were altered in chromatographic mobility after acid treatment, indicating that the α-isoprene unit was not unsaturated (14, 15).

Dolichyl Phosphate—The major product formed after wheat germ acid phosphatase treatment of putative labeled dolichyl phosphate (V) had the mobility of dolichol. To test for the possible presence of unsaturated didehydrodolichyl phosphate, both the insect dolichyl phosphate fraction (V) and authentic 14C-labeled dolichyl phosphate were treated with mild acid and then analyzed by TLC in solvent system B. Neither compound was modified by acid treatment, indicating the absence of a phosphate ester of an allylic alcohol (14, 15).

Mannosyl- and Glucosylphosphoryldolichol—The metabo-
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Ammonium Acetate

B

50mM lOOrM 200mM

U

0 10 20 30 40

Fraction Number

FIG. 1. A, DEAE-cellulose column chromatography of CHCl₃:CH₃OH extract. The CHCl₃:CH₃OH extract from 10⁶ to 10⁷ cells (10⁷-10⁶ cpm) was loaded on a DEAE-cellulose column in [3H]mevalonate-labeled CHCl₃:CH₃OH. Arrows indicate the point of stepwise elution with 50, 100, and 200 mM ammonium acetate in CHCl₃:CH₃OH. B, TLC in solvent system A of the components that did not bind to DEAE-cellulose. The bars indicate the position of migration of authentic standards. C, TLC in solvent system B of the fraction that bound to DEAE-cellulose. The charged fraction was back-washed with water and concentrated prior to TLC analysis. Dol, dolichol.

Dol, dolichol.\(\text{lu}t\text{e IV (Fig. 1C) was tentatively identified as glycosylphosphoryldolichol because it behaved as a negatively charged compound on DEAE-cellulose and had a molecular size consistent with that of a dolichol derivative when subjected to gel filtration on Fractogel TSK-HW 40 (data not shown). To identify the dolichyl phosphate moiety of the putative glycosylphosphoryldolichol, compound IV was subjected to mild acid treatment. As shown in Fig. 3A (lane 1), the radioactive product of acid hydrolysis had the same mobility as dolichyl phosphate. To obtain a preliminary indication of the type of linkage, i.e., a phosphodiester or pyrophosphodiester bond, the mobility of compound IV was compared with that of authentic samples of [\(^3\text{H}\)]mannosylphosphoryldolichol and a mixture of \(N\)-acetyl\([\(^3\text{H}\)]\)glucosaminylpreglucosaminyldolichol and \(N,N'\text{-diacyt}l[\(^3\text{H}\)]\)chitobiosylpreglucosaminyldolichol. Authentic \(N\)-acetyl\([\(^3\text{H}\)]\)glucosaminylpreglucosaminyldolichol and \(N,N'\text{-diacyt}l[\(^3\text{H}\)]\)chitobiosylpreglucosaminyldolichol migrated slower than authentic dolichyl phosphate upon TLC in solvent system B (conditions under which all the phosphate groups are fully charged), whereas authentic \([\(^3\text{H}\)]\)mannosylphosphoryldolichol and compound IV migrated faster than authentic dolichyl phosphate in this solvent, suggesting the presence of a phosphodiester bond in compound IV. To identify the sugar moiety in the putative glycosylphosphoryldolichol, the metabolite was compared with authentic \([\(^3\text{H}\)]\)mannosylphosphoryldolichol by TLC in solvent system B. Surprisingly, the unknown metabolite was found to consist of two radioactive bands (Fig. 3A, lane 2, and Fig. 3B, lane 1), with the lower, faint band corresponding in mobility to \([\(^3\text{H}\)]\)mannosylphosphoryldolichol (Fig. 3A, lane 3). As shown in Fig. 3B (lane 2), the upper, major band of compound IV was found to coincide with authentic \([\(^3\text{H}\)]\)glucosylphosphoryldolichol. As shown in lane 4, [\(^3\text{H}\)]mannosylphosphoryldolichol and [\(^3\text{H}\)]glucosylphosphoryldolichol are separated from each other in this system using solvent system B. The ability to separate mannosylphosphoryldolichol from glucosylphosphoryldolichol by TLC, with the latter migrating faster than the former, has been reported (16). To confirm the identity of the insect cell mannosylphosphoryldolichol, cells were labeled with \([\(^3\text{H}\)]\)mannose in glucose-free Echalar's medium. The TLC profile of the fraction that bound to DEAE-cellulose is shown in Fig. 4. The mobility of the fastest moving mannose-labeled compound (lane 3) corresponded to that of the minor peak obtained with mevalonate labeling (lane 1); this conclusion was confirmed when...
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FIG. 3. TLC in solvent system B of insect cell glycosylphosphoryldolichol (peak IV shown in Fig. 1C). A, Lane 1, product formed by mild acid treatment of glycolipid; Lane 2, glycosyl lipid; Lane 3, mixture of glycosyl lipid and authentic [3H]mannosylphosphoryldolichol; Lane 4, [3H]mannosylphosphoryldolichol. B, Lane 1, glycosyl lipid; Lane 2, mixture of glycosyl lipid and authentic [3H]glucosylphosphoryldolichol; Lane 3, [3H]glucosylphosphoryldolichol; Lane 4, mixture of [3H]glucosylphosphoryldolichol and [3H]mannosylphosphoryldolichol; Lane 5, [3H]mannosylphosphoryldolichol. Dol, dolichol.

FIG. 4. TLC of the [3H]mannose- and [3H]mevalonic acid-labeled compounds in the CHCl₃:CH₃OH fraction that bound to DEAE-cellulose. To two 400-ml suspension cultures of K cells (2-3 × 10⁶ cells/ml) in glucose-free Echler's medium containing 1% fetal calf serum were added 2 mCi of [3H]mannose or 106 μCi of (R)-[5-3H]mevalonic acid. After 4 h, the cells were harvested and the lipids were extracted as described under “Experimental Procedures.” The CHCl₃:CH₃OH (2:1) extract was resolved into bound and unbound fractions by DEAE-cellulose column chromatography and the charged components were subjected to TLC in solvent system B. Lane 1, [3H]mevalonic acid-labeled compounds; Lane 2, mixture of [3H]mevalonic acid- and [3H]mannose-labeled compounds; Lane 3, [3H]mannose-labeled compounds. Dol, dolichol.

A mixture of the two were analyzed (lane 2). It is noteworthy that there was no significant difference in the relative amounts of mevalonate-labeled glycosylphosphoryldolichol and dolichyl phosphate synthesized in glucose-free and in regular Echler's medium (Fig. 4, lane 1, and Fig. 1C). In a separate experiment, cells were labeled with [3H]galactose, which can serve as a precursor of UDP-[3H]Glc in other cells. Analysis of the charged lipids by TLC revealed the presence of a product with the mobility of authentic glucosylphosphoryldolichol (data not shown). Taken together these results indicated that peak IV consisted of a mixture of glucosylphosphoryldolichol and mannosylphosphoryldolichol, with the former being the major component.

N-Acetylglucosamine-containing Dolichol Intermediates—Neither N-acetylglucosaminylpyrophosphoryldolichol nor N,N'-diacetylchitobiosylpyrophosphoryldolichol could be detected in the fraction that bound to DEAE-cellulose, as shown in Fig. 5. To exclude the possibility that these two lipids were formed but degraded during extraction, a mixture of authentic 3H-labeled glucosaminylpyrophosphoryldolichol and N,N'-diacetylchitobiosylpyrophosphoryldolichol was added to a pellet of K cells. The cell pellets were then immediately extracted with CHCl₃:CH₃OH and the extract was chromatographed on a DEAE-cellulose column as described under “Experimental Procedures.” Over 95% of the added radioactivity was recovered in the CHCl₃:CH₃OH extract. Moreover, greater than 90% of the radioactivity in the CHCl₃:CH₃OH extract bound to the DEAE-cellulose column and was subsequently eluted with ammonium acetate.

Characterization of Metabolites Extractable with CHCl₃:CH₃OH:H₂O

The putative labeled oligosaccharide-lipid derived from [3H]mevalonic acid and recovered in the CHCl₃:CH₃OH:H₂O fraction was subjected to silica gel TLC in solvent system C. It exhibited a single radioactivity peak with an Rf value of 0.16. The compound did not migrate from the origin in solvent systems B or G. The putative oligosaccharide-lipid had the...
same chromatographic mobility in solvent system C as [14C] isopentenyl pyrophosphate; however, unlike this compound, it was resistant to alkaline phosphatase treatment. When it was treated with mild acid (0.1 M HCl, 50 °C, 30 min), two radioactivity peaks that migrated faster than the starting compound were observed upon TLC in solvent C. The faster moving one coincided in mobility to dolichyl phosphate; the slower moving one was tentatively identified as dolichyl pyrophosphate because 1) this chromatographic system separated polisoprenyl monophosphate from polisoprenyl pyrophosphate (15) and 2) stronger acid treatment (0.1 M HCl, 95 °C, 30 min) increased the amount of dolichyl phosphate and decreased the amount of putative dolichyl pyrophosphate. The insect oligosaccharylpyrophosphoryldolichol bound to DEAE-cellulose equilibrated with CHCl₃:CH₃OH:H₂O and eluted more slowly than authentic [3H]mannosylphosphoryldolichol with a linear gradient of 0–0.1 M ammonium acetate in CHCl₃:CH₃OH:H₂O.

To support the conclusion that the product isolated in the CHCl₃:CH₃OH:H₂O fraction was oligosaccharylpyrophosphoryldolichol, authentic [14C]glucose-labeled oligosaccharylpyrophosphoryldolichol was synthesized in vitro in hen oviduct microsomes in the presence of UDP-N-acetylgalactosamine, GDP-mannose, UDP-[14C]glucose, and dolichyl phosphate (10). In Fig. 6 are shown the TLC profiles of a mixture of insect cell [3H]-labeled oligosaccharylpyrophosphoryldolichol and authentic [14C]glucose-labeled oligosaccharylpyrophosphoryldolichol before and after treatment with 0.1 M HCl at 95 °C for 30 min. Before acid hydrolysis (Fig. 6A), both the [3H]-labeled compound and [14C]oligosaccharylpyrophosphoryldolichol migrated with the same mobility, although the insect preparation contained an additional minor component of high mobility that probably is a breakdown product. TLC analysis (Fig. 6B) after acid treatment revealed that the majority of the [3H]-labeled compound then migrated with a mobility identical to dolichyl phosphate, whereas the [14C]-labeled product, as expected for a free oligosaccharide, remained at the origin.

Dynamics of [3H] Mevalonic Acid- and [3H] Mannose-labeled Dolichol Intermediates

Having characterized the major dolichol-linked intermediates synthesized by Kc cells, we turned to measurements of their metabolism. The results of a pulse-chase experiment are summarized in Fig. 7. As shown in panel A, following short term labeling (4 h) when it is unlikely that the steady state has been achieved, one of the major products, dolichyl phosphate, apparently is converted to dolichol during the chase. Overall, the total amount of labeled dolichol derivatives remained constant. After long term labeling (panel B), the relative levels of each of the intermediates is quite different from that shown in panel A. Moreover, relatively little change in their levels occurred during the chase. The relative levels of the intermediates during short term (4-h) and steady state (4-day) labeling are summarized in Table I. It is evident from the chase experiments that the cellular level of the glycosyl dolichol derivatives remained relatively constant. If these compounds are involved as saccharide donors, one would expect that, even if the dolichol backbone is stable, the glycosyl moiety should exhibit rapid turnover. To test this idea, Kc cells were pulse-labeled with [3H]mannose and the mannosylated lipids were analyzed by TLC over the course of the chase. The results of this experiment revealed a dramatic decrease in the amount of [3H]mannosylphosphoryldolichol observed during the chase (Fig. 8A). An unknown, charged metabolite derived from [3H]mannose, which was not labeled with mevalonic acid and therefore does not contain dolichol, decreased more slowly over the course of the chase. Quantitative analysis of the turnover of the mannosyl moiety of mannosylphosphoryldolichol indicated that its half-life (t½) was 3 h (Fig. 8B).

Effect of Ecdysone and Tunicamycin on Dolichol-related Intermediates

Although the Kc cells responded to ecdysone (the molting hormone) by undergoing morphological changes identical to...
FIG. 7. Kinetics of synthesis and turnover of dolichol derivatives. A, Kc cells (2 × 10⁶ cells/ml, 500 ml) were prelabeled with 250 μCi of [³H]mevalonic acid for 4 h, harvested, washed, and then incubated for 0, 8, 18, and 30 h in nonradioactive Eschalier's medium containing 1% fetal calf serum. B, Kc cells (7 × 10⁶ cells/ml, 400 ml) were prelabeled with 400 μCi of [³H]mevalonic acid for 4 days, harvested, washed and then incubated for 0, 12, 24, and 36 h in nonradioactive Eschalier's medium containing 1% fetal calf serum. The CHCl₃:CH₃OH extract of the cells was analyzed by DEAE-cellulose chromatography followed by silica gel TLC as described under "Experimental Procedures." O——O, glycosylphosphoryldolichol (Glycosyl-P-Dol); D——D, dolichyl phosphate (Dol-P); Δ——Δ, dolichol (Dol); ▲——▲, oligosaccharilypyrophosphoryldolichol (Oligo-PP-Dol).

TABLE I
Composition of dolichol and its derivatives from Drosophila Kc cells labeled with [³H]mevalonic acid

The Kc cells were labeled with [³H]mevalonic acid as described in the legend to Fig. 7.

| Metabolites | 4-h labeling | 4-day labeling |
|-------------|--------------|---------------|
|             | Radioactivity | Mole ratio | Radioactivity | Mole ratio |
|             | cpm x 10⁻³    |           | cpm x 10⁻³    |           |
| Dol-P       | 162           | 1.00      | 203           | 1.00      |
| Dol         | 91            | 0.56      | 1241          | 6.11      |
| Chito-PP-Dol| ND            | <0.05     | <33           | <0.16     |
| Glc-P-Dol   | 74            | 0.46      | 1285          | 6.33      |

*The radioactivity found in coenzyme Q after labeling for 4 h or 4 days was 648,000 and 3,219,000 cpm, respectively.

**Not detectable.

those observed in other reports (17–20), in the presence of this hormone no change in the synthesis of dolichol derivatives or in coenzyme Q (Table I) was observed. Tunicamycin had little effect on coenzyme Q synthesis but, as expected, oligosaccharilypyrophosphoryldolichol synthesis was inhibited. It was surprising, however, that this drug caused an actual increase in total incorporation into the total dolichol-related intermediates (Table II). A more detailed study (data not shown) of the dose response of Kc cells to tunicamycin established that the maximum effect was observed at the tunicamycin concentration (1 μg/ml) used in the experiment shown in Table II. As the concentration of tunicamycin was increased from 0 to 1 μg/ml, incorporation into dolichyl phosphate remained nearly constant, whereas incorporation into both mannopolyporphoryldolichol and glucosylphosphoryldolichol progressively increased (3- and 2.1-fold, respectively). As expected, the amount of oligosaccharilypyrophosphoryldolichol decreased. These results suggest that the labeled dolichyl phosphate that accumulates as a result of inhibition of its conversion to N-acetylgalactosaminopropene-
phosphoryldolichol by tunicamycin is utilized in the formation of glycosylphosphoryldolichol, especially mannosylphosphoryldolichol. However, the mechanism whereby the total incorporation into these dolichol derivatives is increased in the presence of tunicamycin is not clear.

**Uptake of $[^{14}C]$Dolichol into Cultured Cells**

Cells (2.9 x 10^6 cells/ml) were incubated with 30uCi of $[^{14}C]$dolichol during a 4-day period. Cells were then harvested, washed with Eschallier's medium four times, and then extracted with CHCl₃:CH₃OH and with CHCl₃:CH₃OH:H₂O as described under "Experimental Procedures." As a control, an equivalent amount of $[^{14}C]$dolichol was added to cells that were immediately harvested and extracted with CHCl₃:CH₃OH and with CHCl₃:CH₃OH:H₂O. Analysis by DEAE-cellulose chromatography, followed by silica gel TLC in solvent system B and radioautography, revealed that exogenous $[^{14}C]$dolichol had been converted to dolichol phosphate, glucosylphosphoryldolichol, and mannnosylphosphoryldolichol. Although only 0.1% of the radioactivity recovered in the CHCl₃:CH₃OH fraction was present in the form of phosphorylated derivatives, this finding indicated that K cells contain a kinase capable of phosphorylating free dolichol.

**Do Novo Synthesis of N Linked Glycoproteins**

To confirm that under the conditions of these in vivo experiments the K cells were involved in assembly of N-linked glycoproteins, the proteins from cells labeled with either $[^{3}H]$glucosamine or $[^{3}H]$mannose were precipitated with trichloroacetic acid and digested with Pronase. When the resulting glycopeptides were analyzed by Sephadex G-50 gel filtration three classes of labeled materials were separated; $V_p$ primarily consisting of glycosaminoglycans excluded from the column (21, 22), glycopeptides with a $V_p/V_o$ of 1.8, and an included fraction ($V_I$). In the case of $[^{3}H]$glucosamine labeling, 37% of radioactivity in the Pronase digest was associated with the glycopeptide fraction, whereas in the case of $[^{3}H]$mannose, 76% of the radioactivity was found in the glycopeptide fraction. The two labeled glycopeptide fractions recovered from the gel filtration column were analyzed further on a concanavalin A-agarose column. About 80% of each of the glycopeptide fractions was bound to this lectin, indicating that they both contained a-mannosyl units in their oligosaccharide chains.

**DISCUSSION**

In a series of studies Watson and his co-workers (3-6) have demonstrated that a variety of insect cell lines, including *Drosophila* K cells, do not synthesize squalene or sterols. The major nonammonifiable products synthesized from mevalonate in this cell line were shown to be coenzyme Q and dolichol; analysis for polar derivatives of dolichol was not carried out. Studies in a mosquito cell line showed that polymannose N-linked glycoproteins are present (1) and that their assembly appeared to be mediated via dolichol intermediates (2). Based on these observations and the likelihood that studies on synthesis of dolichol and its derivatives would be facilitated by the absence of squalene and cholesterol biosynthesis, we undertook to determine if cultured K cells would provide new insights into dolichol metabolism and the dolichol-linked pathway for glycoprotein synthesis. These cells are an established line of unknown tissue origin that was isolated from *Drosophila* embryos and shown to be responsive to the steroid hormone ecdysone (20). Cells were labeled with $[^{3}H]$mevalonic acid and the labeled lipids, isolated by sequential extraction with CHCl₃:CH₃OH and CHCl₃:CH₃OH:H₂O, were characterized by a variety of chromatographic and chemical methods described under "Results." The CHCl₃:CH₃OH-extractable products were separated into charged and uncharged lipids. The uncharged lipids were shown to be coenzyme Q, tarsenol, and dolichol; in agreement with an earlier study (3), no fatty acid esters of dolichol were found. The major charged isoprenoid compounds were shown to be dolichyl phosphate, glucosyldolichol, and mannnosyphosphoryldolichol. No labeled polyisoprenoid derivatives corresponding to mobility to N-acetylglucosaminylphosphoryldolichol or N,N'-diacetylchitobiosylphosphoryldolichol were detected. In the CHCl₃:CH₃OH:H₂O fraction, the major labeled product was found to be oligosaccharylphosphoryldolichol.

Having identified the isoprenoid products of metabolic labeling, we undertook to estimate the relative level of each during short and long term labeling. It is important to note that these labeling experiments provide a true estimate of the relative amounts of the labeled dolichol derivatives because all arise from a common precursor. This is in marked contrast to the many studies carried out over the past decade using sugar labeling of glycosyl moieties attached to dolichol. Quantification of these sugar-labeled intermediates has been difficult because of possible differences in (a) the rate of sugar uptake and (b) the pool sizes of the different sugars and sugar nucleotides. Under these circumstances the relative levels of the different radioactive glycosylated derivatives of dolichol may have little resemblance to the actual amount synthesized.

Using mevalonic labeling, dramatic differences in the relative amounts of the labeled intermediates was observed between short and long term labeling. After 4 h of labeling, the major products were glucosylphosphoryldolichol and dolichyl phosphate. However, after long term labeling (4 days), glycosylphosphoryldolichol, oligosaccharylphosphoryldolichol, and dolichol were all present at approximately equivalent levels and dolichyl phosphate was very low. Under neither labeling condition could significant amounts of N-acetylglucosaminylphosphoryldolichol or N,N'-diacetylchitobiosylphosphoryldolichol be detected. The rate and extent of loss of dolichyl phosphate corresponded to the rate and the extent of increase in dolichol, suggesting that dolichyl phosphate is the precursor of dolichol. After either short or long term labeling, the total level of labeled polyprenoid chains did not decrease during the chase, indicating the absence of a major catabolic pathway. Similar stability was observed in the level of glycosylphosphoryldolichol. However, this finding does not mean that this fraction is static. In fact, when the sugar moiety of the mannosyphosphoryldolichol component was labeled and then subjected to a chase, a half-life of approximately 3 h was observed. It is clear, then, that this pool is in a metabolically active state with respect to the glycosyl unit, although the total amount of glycosylphosphoryldolichol remains constant. This, coupled with the fact that the level of free dolichyl phosphate only slowly declined over a 30-h period, suggests that the dolichyl phosphate moiety in mannosyphosphoryldolichol is rapidly reutilized for mannoxylation.

These results, which for the first time in any biological system provide an estimate of the relative levels of a variety of newly synthesized dolichol derivatives, can be considered in the context of the biosynthetic pathway for glycoprotein synthesis shown in Scheme 1. The results suggest that the end product of de novo synthesis from mevalonate is dolichyl phosphate. Some of this dolichyl phosphate is converted to dolichol while the rest is used for oligosaccharide-lipid assembly. Much earlier work has shown that assembly of oligosaccharylphosphoryldolichol is initiated by conver-
sion of dolichyl phosphate to \( N \)-acetylglucosaminylpyrophosphoryldolichol (23). However, no \( N \)-acetylglucosaminylpyrophosphoryldolichol or \( N,N' \)-diacetychitobiosylpyrophosphoryldolichol could be detected by mevalonic acid labeling of \( K \), cells under conditions in which mannosylphosphoryldolichol was readily detected. Two observations make it unlikely that the reason for turn over rapidly, as expected if oligosaccharide chains are being assembled. Thus, in reference to the pathway shown in Scheme 1, although the level of the initial acceptor (dolichyl phosphate) is low relative to the two glycosylphosphoryl lipids and the oligosaccharyl lipid, the level of all of these compounds must be high relative to the \( N \)-acetylglucosamine-containing intermediates. This suggests that these early \( N \)-acetylglucosaminyl intermediates never have a chance to build up, presumably because the flux through the early steps in this pathway is rapid. Perhaps the much higher levels of the mannosyl and glucosyl derivatives serve to ensure that incomplete saccharide chains of intermediate size do not accumulate and thus would not be available for transfer to protein.

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**SCHEME 1.** MVA, \(^{[3H]}\)mevalonic acid; Dol-P, dolichyl phosphate; Dol, dolichol.