Intracellular trafficking of dolichol: on the presence of dolichol transfer activity in bovine liver supernatant*

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Key words: dolichol, lipid transfer protein, bovine liver, intracellular lipid flow

A protein catalyzing dolichol transfer between membranes has been purified from bovine liver up to 600-fold by acid precipitation, ammonium sulfate precipitation, ion-exchange chromatography and hydrophobic interaction chromatography. The protein displays a relative molecular mass of 15000 on SDS-gel electrophoresis. Kinetics as well as the influence of a series of effectors were studied. The transfer activity is inhibited by sphingomyelin, sulfhydryl groups and cationic amphiphilic amines with a bulky heterocyclic aromatic function. High salt concentration decreases the transfer efficiency. Transfer of dolichol between vesicles and mitochondria is not affected by the presence of moderate amounts of cholesterol in the donor vesicles. The overall characteristics of dolichol transfer activity are discussed in comparison to those of other lipid transfer proteins.

Dolichols are long chain cis-polyisoprenols virtually present in all eukaryotic cells. They are found as free alcohols and esterified with long-chain fatty acids. A minor portion is (pyro)phosphorylated and eventually conjugated with carbohydrates [1]. A novel ester derivative, dolichyl dolicholate, has been identified in bovine thyroid [2]. The biosynthesis of dolichol is mainly confined to the endoplasmic reticulum, where the phosphorylated derivative is supposed to exert its biological function as a cofactor in the N-glycosylation of proteins [3].

On studying the subcellular fate of de novo synthesized dolichol an intracellular flow was found to occur [4, 5]. In addition several authors have casually referred to the occurrence of non-membrane associated supernatant dolichol [6–11]. Recently Van Dessel et al. [12], reporting on dolichol binding capacity by a bovine liver pH 5.1 supernatant, have hypothesized the existence of a dolichol transporting system as already described for the intracellular transfer of other lipids [13, 14].

With regard to intracellular dolichol trafficking from the site of synthesis to other membranes only a rudimentary picture exists concerning the mechanism involved. Vallersson et al. [15] have suggested that conjugation with specific fatty acids might exert a signalling function for targeting dolichol to its final subcellular destination.

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1 Abbreviations: Ch, cholesterol; CHE, cholesteryl esters; Dol, dolichol; Dol-TA, dolichol transfer activity; ER, endoplasmic reticulum; GL, glycolipids; LTP, lipid transfer protein; nL-TP, non-specific lipid transfer protein; P, particulate fraction; FC, phosphatidylycholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PMSF, phenylmethylsulfonyl fluoride; S, 105000 × g supernatant; SUV, small unilamellar vesicles; SQ, squalene; TG, triglycerides; α-Toc, α-tocopherol.
The present report aims to provide a more solid experimental evidence for the existence of a dolichol transferring factor in bovine liver.

MATERIALS AND METHODS

Phenyl-Sepharose and Concanavalin A Sepharose 4B were acquired from Pharmacia. Papain, pronase and trypsin were from Boehringer Mannheim. $[^3]$H]Dolichol (6.6 Ci/mmol), $[^14]$Clabeled phosphatidylcholine (122 mCi/mmol), $[^14]$Clabeled phosphatidylethanolamine (33 mCi/mmol) and $[^14]$C-cholesterol (60 mCi/mmol) were obtained from Amersham. Non-labeled lipids were purchased from Sigma and/or Supelco. The purity of the lipids was checked by TLC using standard solvent systems. $[^3]$H-Dolichyl monophosphate was synthesized from $[^3]$H-Dolichol as described by Steen et al. [16].

Lipid vesicles. SUV's, used as donor vesicles in these studies, were prepared by sonication. Briefly, 3 μmol phosphatidylcholine, 0.5 μCi $[^3]$H-dolichol and 0.1 μCi $[^14]$C-cholesterol (a non-exchangeable marker) were mixed in chloroform/methanol (1:1, v/v). After evaporation of the solvent under a nitrogen stream the lipid residue was dried under vacuum for 2 h. SET-buffer (1 ml 0.25 M sucrose/1 mM EDTA/50 mM Tris, pH 7.4/0.02% NaN3) was added and the suspension was vortexed thoroughly. The multilamellar dispersion was sonicated for 6 min with intermittent cooling (0-4 mm probe; Braun Sonic 300 sonifier). The clear dispersion was centrifuged at 20000 × g for 20 min to remove titanium particles and multilamellar liposomes. The upper 2/3 of the supernatant was used as SUV's source within 48 h.

Mitochondria. Bovine thyroid mitochondria were prepared as described previously [17], and stored at −20°C. After thawing they were heated for 30 min at 60°C and washed three times with SET-buffer. Assay of dolichol transfer activity. Typically the assay mixture consisted of SUV's (50-60 nmol phospholipids), mitochondrial acceptor fraction (600 nmol phospholipid) and transfer fraction (equivalent to 100-250 μg protein) in a total volume of 0.5 ml SET-buffer [18]. The transfer assays were terminated after 15 min at 37°C by centrifugation at 17600 × g for 5 min. The pellet was washed once with SET-buffer and after recentrifugation counted in a water-lumosolve-lipoluma cocktail (0.2:1:9; by vol.) [19]. The amount of protein-mediated transfer equals the total transfer minus the sum of transfer in the absence of protein fraction and transfer in the absence of acceptor fraction. The transfer activity was expressed in pmoles dolichol/mg protein per h.

Amphiphilic amines were added directly to the assay mixture, just prior to incubation, from ethanolic solutions (the final ethanol concentration never exceeded 1%).

Purification of proteins capable of transferring dolichol. The purification scheme of bovine liver Dol-TA was patterned after that developed for rsl-TP [18]. Starting from a pH 5.1 supernatant a purification of about 40-fold with an overall yield of 35% for Dol-TA was obtained through: i, ammonium sulfate precipitation; ii, cation exchange (CM-cellulose) chromatography and iii, heat treatment. Further purification was performed either on phenyl-Sepharose or on Concanavalin A-Sepharose 4B.

Hydrophobic interaction chromatography on phenyl-Sepharose (2 cm × 9 cm) was used instead of octyl-agarose as originally proposed by Crain & Zilversmit [18]. Then the heat stable fraction was adjusted to 4 M NaCl with solid NaCl. The proteins were eluted first with 0.15 mM NaCl until A280 < 0.3 and then with water; fractions of 1 ml were collected. Before monitoring the transfer activity in the salt elute, the fractions were dialyzed against SET-buffer.

As an alternative, the heat stable protein fraction was subjected to Con A affinity chromatography. The sample (500 μg protein) was applied to a Concanavalin A-Sepharose column (1 cm × 10 cm) and unbound proteins were eluted with 20 mM Hepes/0.5 M NaCl/1 mM MnCl2/1 mM CaCl2, pH 7.4. The retained proteins were eluted with the same buffer supplemented with 0.5 M α-methyl mannoside. Fractions of 2 ml were collected.

Dolichol kinase assay. Dolichol kinase activity in bovine thyroid microsomes was assayed as described previously [20] in the absence and presence of Dol-TA equivalent to 35 μg protein.

Other analytical methods. Polycrylamide gel electrophoresis under denaturing conditions on 7.5% acrylamide gels was performed using standard techniques [21]. The relative molecular mass of the transfer factor was read.
from a log10 relative molecular mass vs relative mobility plot (low molecular weight standards Pharmacia). Protein was determined by the method of Lowry et al. [22] after precipitation of the proteins with 20% trichloroacetic acid. Lipid phosphorus was measured as described earlier [23].

RESULTS

Purification of dolichol transfer activity

Employing the purification protocol for the nsL-TP up to and including the heat treatment, dolichol transfer activity factor could be shown in those fractions also containing nsL-TP (Table 1). However, upon hydrophobic interaction chromatography of the Dol-TP positive fractions an elution pattern differing from that of nsL-TP was observed. Dolichol transfer activity could be detected in both eluates (Fig. 1A). Crain & Zilversmit [18] on the other hand, could only demonstrate LTP activity in the water eluate. The highest purification and yield for Dol-TP were obtained in the low ionic strength eluate (Table 1). This most purified protein fraction (enrichment factor 60) was able to transfer 37 pmoles dolichol/mg protein per h vs 21 pmoles/mg protein per h in the high salt eluate. Monitoring the $^{14}$C phosphatidylcholine transfer activity in the low ionic strength eluate showed a recovery of 20% with an enrichment factor of 937 and a specific activity of 123 n mole/mg protein per h in agreement with Crain & Zilversmit [18]. Phosphatidylethanol-amine was also transferred at a comparable rate.

The phenyl-Sepharose fractions were very labile and lost their dolichol transfer activity after 48 h at 4°C. Addition of 10% glycerol gave a slight improvement in stability upon storage. On SDS-gel electrophoresis only one band with a relative molecular mass of 15000 was seen (Fig. 1B).

Other attempts (DEAE-Sephadex A50, Sephadex G75, hydroxyapatite) to improve the purification were unsuccessful. Applying the heat stable protein fraction on Con A-Sepharose resulted into an elution profile with two protein peaks. The transfer activity, emerging always just in front of the second protein peak which was chased from the column with α-methylmannopyranoside containing eluent, was much lower (specific activity 5.3 pmoles/mg protein per h) (Fig. 2) than that measured in the phenyl-Sepharose eluate. On non-substituted Sepharose 4B this retarding effect was not observed. So far there is no explanation for the peculiar behaviour of Dol-TP on Con A Sepharose.

Kinetics of the dolichol transfer

Due to the great instability of the purified protein, most incubations were performed with the heat stable protein fraction. Figure 3A illustrates the contribution of each component of the transfer assay (15 min incubation point) as affected by the composition of the incubation medium. Even in the absence of acceptor fraction low levels of radioactivity can be spun down. In the absence of transfer factor but including the acceptor fraction much higher levels of radioactivity can be sedimented due to spontaneous transfer. When all three components are included, the transfer rate is appreciably enhanced, supporting the stimulatory

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein mg</th>
<th>Specific activity pmoles/mg protein per h</th>
<th>Recovery %</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.1 supernatant</td>
<td>2990</td>
<td>0.06</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitation (50%–90%)</td>
<td>328</td>
<td>0.5</td>
<td>91</td>
<td>8</td>
</tr>
<tr>
<td>CM-cellulose + 90°C treatment</td>
<td>25</td>
<td>2.4</td>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>Phenyl-Sepharose: salt eluate</td>
<td>0.69</td>
<td>20.7</td>
<td>8</td>
<td>345</td>
</tr>
<tr>
<td>water eluate</td>
<td>0.55</td>
<td>37</td>
<td>11</td>
<td>671</td>
</tr>
</tbody>
</table>

Table 1

Purification of dolichol transfer activity from bovine liver.

Average of at least three determinations.
Fig. 1. Chromatography of Dol-TA on phenyl-Sepharose.
A. Elution profile on phenyl-Sepharose. Conditions: see Materials and Methods. Fractions of 2 ml were collected. The arrow indicates the change of eluent to water. Transfer activity assays were performed as described under Materials and Methods (0.2 ml/fraction). O, A280 nm; hatched area, dolichol transfer activity. B. SDS-gel electrophoretic pattern (scanned at 610 nm) of the phenyl-Sepharose water eluate.

Fig. 2. Chromatography of Dol-TA on concanavalin A Sepharose 4B.
Conditions: see Materials and Methods. Arrow indicates the change of eluent to 0.5 M α-methylmannopyranoside. Transfer activity assays were performed as described under Materials and Methods. O, A280 nm; Δ, transfer activity.

effect of the transfer factor. In Fig. 3B the time course of the net dolichol transfer is represented showing linearity up to about 10 min. In function of incubation time, spontaneous transfer also gradually increases but to a lesser extent. Supplementation of the incubation buffer with 10% glycerol promoted the transfer activity (1.6 fold) while additionally the linearity vs time was maintained for a longer period (up to 30 min). Figure 3C shows a linear relationship between the percentage of dolichol transfer and the amount of dolichol transfer factor up to 250 μg. The rate is optimal at pH 8 (Fig. 3D), although between pH 8 and 9 no drastic alteration of transfer activity is seen. From experiments with donor liposomes loaded with dolichyl monophosphate it became obvious that the transfer factor is also able to translocate this dolichyl derivative (Fig. 4), however at a much slower rate.

Influence of effectors

Table 2 shows that the transfer activity is sensitive towards thiol group reagents. Amphiphilic amines also influence the level of dolichol transfer (Table 3). Most potent inhibition was perceived with the cationic amines propranolol and chloroquine. Increase in the ionic strength was accompanied by an inhibition of the dolichol transfer activity (Fig. 5). Diverging effects could be noted depending on the salt type administered, cadmium-salts being the most effective.

A series of assays was performed using liposomal preparations with varying lipid composition. The results of these experiments with egg PC-liposomes as a reference donor system have been summarized in Table 4. From these data it is evident that the rates of dolichol transfer were dependent on the nature of the matrix lipid, its charge and degree of unsaturation.

Table 5 demonstrates that the transfer activity was protease-sensitive. When BSA is included instead of the transfer protein in the incubation mixture, no protein-mediated dolichol transfer can be measured.

Physiological function

When dolichol kinase was assayed in a bovine thyroid microsomal fraction in the presence of Dol-TA, the activity was 1.3 fold enhanced.
DISCUSSION

Since \textit{de novo} biosynthesis of phospholipids, cholesterol and triglycerides occurs in the endoplasmic reticulum and the newly synthesized lipids are readily distributed throughout the cellular membranes it is believed that specific as well as non-specific lipid transfer proteins play a crucial role in this intracellular trafficking [24]. With the exception of peroxisomes which possess their own biosynthetic machinery [25] dolichol synthesis also takes place mainly in the ER [26] from where it is distributed to the other cellular compartments. In addition, as recently reported, newly synthesized dolichol can also partially be secreted into the bile and blood by (an) as yet unknown mechanism(s) [27]. From biochemi-
Table 2

Influence of thiol agents on the dolichol transfer activity.
Transfer activity was monitored under standard conditions (123 µg of protein, 15 min) except that the protein fraction was preincubated with the thiol agents (20 min, 37°C) at the concentration indicated prior to the transfer assay.

<table>
<thead>
<tr>
<th>Thiol agent</th>
<th>Concentration (mM)</th>
<th>Dolichol transferred* (%) versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.2</td>
<td>49 ± 7</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>0.2</td>
<td>44 ± 7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>19 ± 11</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>5</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>Aldithiol-4</td>
<td>0.2</td>
<td>86 ± 5</td>
</tr>
</tbody>
</table>

*Average of three experiments.

Table 3

Modulation of dolichol transfer activity by amphiphilic amines
Effectors were added directly to the assay medium just before incubation (200 µg of protein, 15 min).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration (mM)</th>
<th>Dolichol transferred* (%) versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Propranolol</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>1</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>1</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1</td>
<td>93 ± 5</td>
</tr>
</tbody>
</table>

*Average of three experiments.

Fig. 4. Transfer of dolichyl monophosphate.
Transfer activity assays were performed under standard conditions (dolichyl monophosphate: 62000 d.p.m./incubation). Average of duplicate experiments.

...cal logics one could speculate that in these intracellular movements of dolichol (a lipid transfer factor(s) might be involved.

In this paper first evidence is given for the occurrence of a dolichol transfer factor in the postmitochondrial supernatant of bovine liver. Although substantial spontaneous transfer activity does occur, inclusion of the protein factor in the incubation mixture considerably increases the dolichol transfer activity. Appreciable non-protein mediated lipid transfer activity has also been reported by others [28, 29]. Furthermore the protein nature of the transfer activity could be inferred from its sensitivity towards proteolytic agents. The transfer activity was purified following a slightly modified procedure as outlined by Crain & Zilversmit [18] for rsL-TP. In contrast to that reported by Crain & Zilversmit [18] the elution pattern after hydrophobic interaction chromatography displayed a biphasic profile. Whether this observation is an indication of the occurrence of...
Table 4

Influence of composition of donor vesicles on the dolichol transfer activity

<table>
<thead>
<tr>
<th>Donor composition</th>
<th>Transfer ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (egg)</td>
<td>1</td>
</tr>
<tr>
<td>DPPC</td>
<td>0.4</td>
</tr>
<tr>
<td>CH/PC (1/9)</td>
<td>1.1</td>
</tr>
<tr>
<td>DCP/PC (1/9)</td>
<td>2.3</td>
</tr>
<tr>
<td>PA/PC (1/9)</td>
<td>1.4</td>
</tr>
<tr>
<td>F/PC</td>
<td>3.3</td>
</tr>
<tr>
<td>SPH/PC (1/1)</td>
<td>0.02</td>
</tr>
<tr>
<td>(1/9)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*PC, phosphatidylcholine; DCP, dicetylphosphate; DPPC, dipalmityloxyphosphatidylcholine; PA, phosphatidic acid; F, farnesol; SPH, sphingomyelin. Compositions were expressed as mol percent.

**Transfer activities were measured under standard incubation conditions (107 μg of protein, 15 min).

two different proteins with dolichol transfer activity cannot yet be answered. The enrichment factor for Dol-TA amounted to about 600 with a yield of 11%. The purification agrees with the enrichment found for nsL-TP isolated by Crain & Zilversmit [18]. The final Dol-TA preparation was almost pure as judged by polyacrylamide gel electrophoresis. The migration distance corresponded to a protein with a relative molecular mass of 15000, very close to that reported for nsL-TP [30].

The dolichol transfer factor displays several characteristics (Table 6) very similar to those of the nsL-TP from bovine liver [18]: i, both transfer activities withstand heat treatment; ii, dolichol transfer activity is sensitive to the action of sulphhydryl reagents as reported for other lipid transfer proteins [31]. The possible involvement of essential thiol groups also fits the observation that the dolichol transfer is severely decreased in the presence of Cd**-ions; iii, the stimulatory effect of dicetylphosphate and phosphatidic acid has also been reported for protein-mediated PC transport [32]; iv, the salt sensitivity of the dolichol transfer is also a recurrent theme supporting a role for ionic interactions in the transfer process [32]; v, Dol-TA is also able to displace phospholipids (PC, PE) from donor vesicles to a mitochondrial acceptor. However, for dolichol much lower transfer velocities are registered. This probably results from substrate specificity. One should keep in mind that the molecular dimensions of dolichols and phospholipids differ appreciably.

Table 5

Sensitivity of dolichol transfer activity to proteolytic inactivation.

Dolichol transfer activity (1 mg of the heat stable protein) was incubated with 200 μg protease. The proteolytic activity was inhibited by addition of either trypsin inhibitor or PMSF, whereafter the transfer activity was measured as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Percent activity versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% activity in control*</td>
</tr>
<tr>
<td>Trypsin</td>
<td>79</td>
</tr>
<tr>
<td>Papain</td>
<td>87</td>
</tr>
<tr>
<td>Pronase</td>
<td>84</td>
</tr>
</tbody>
</table>

*The control samples were treated the same way with the omission of protease.
Table 6

Characteristics of intracellular lipid transfer proteins.

<table>
<thead>
<tr>
<th>Subcellular localization</th>
<th>TC/CHE</th>
<th>α-Tocopherol</th>
<th>αsL-TP (SCP2)</th>
<th>Squalene (SPF)</th>
<th>PC</th>
<th>PL</th>
<th>Dol-TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per (lumen)</td>
<td>P</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Mr.</td>
<td>200000</td>
<td>34000</td>
<td>12800–14800</td>
<td>?</td>
<td>24000</td>
<td>32000</td>
<td>15000</td>
</tr>
<tr>
<td>Protease</td>
<td>?</td>
<td>–</td>
<td>+</td>
<td>?</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ionic strength</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Substrate</td>
<td>TC/CHE</td>
<td>αToc</td>
<td>PC, CH, GL</td>
<td>SQ</td>
<td>PC</td>
<td>PL</td>
<td>PE, PC, Dol</td>
</tr>
<tr>
<td>Activity (pmol/mg protein per h)</td>
<td>18000</td>
<td>84-5700*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37</td>
</tr>
</tbody>
</table>

*Depending on the species under investigation.

The inhibition by amphiphilic amines with a positive and bulky heterocyclic aromatic function is very similar to that reported for bovine brain PC- and PI-transfer protein [33]. Therefore both electrostatic and hydrophobic interactions may be involved in the transfer mechanism.

From Table 4 it follows that the dolichol transfer activity is, at least partially, dictated by the lipid composition of the donor vesicles: i) decreasing the degree of unsaturation more than halved the dolichol transfer (PC vs DPPC vesicles); ii) on the other hand cholesterol, assumed to rigidify membranes above transition temperature and as such expected to hinder lipid removal out of the membrane was without any significant effect. However, according to Johnson & Zilversmit [34] this type of interaction can only be seen at higher cholesterol/phospholipid ratios; iii) introduction of negative charges in the donor vesicles was accompanied by enhanced transfer, incorporation of dicetylphosphate being the most effective; iv) sphingomyelin inhibits the dolichol transfer activity. Several other proteins, interacting with membrane surfaces e.g., phospholipase C (Bacillus cereus) [35] and PC-TP from bovine liver [36] are also known to be affected by the presence of sphingomyelin. The phenomenon might result from a tighter packing of the headgroups (hydrogen belt) and/or a rigidifying effect on the PC matrix [37, 38]. Alternatively a strong interaction between sphingomyelin and dolichol, as described for cholesterol [39], could retard the movement of dolichol out of the membrane.

Despite the wealth of information on structure and activity of several transfer proteins, the in vivo function of these transfer proteins is still not understood. A growing number of papers provide evidence for the involvement of these proteins in numerous enzymatic reactions [1], e.g. SCP2 has been shown to stimulate dolichol biosynthesis [40]. In testing this possibility for Dol-TA with dolichol kinase as an enzyme source, a moderate increase of the phosphorylation activity was observed in the presence of Dol-TA. The amplifying effect was far beneath that seen with other enzyme systems [41].

Another interesting feature of lipid transfer proteins is their application as a tool for the systematic replacement, extraction or insertion of lipids into membranes. This approach allows to ascertain consequences of controlled alteration of membrane lipid composition on functional parameters and can be assessed directly [42]. In this way the effect of dolichol incorporation on the fluidity of native thyroidal membranes has been studied in our laboratory [43].

Summarizing, we have been able to purify a protein factor from bovine liver supernatant accelerating the transfer process of dolichol between vesicles and mitochondria. Although the
transfer factor displays some common characteristics with other LTP, further research will be needed in order to define more precisely its identity, characteristics and mechanism of action.

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