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Study on the Biosynthesis of Dolichol in Yeast: Recognition of the Prenyl Chain Length in Polyprenol Reduction[1]

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We synthesized three water-soluble biotin-tagged compounds with different prenyl chain lengths, biotinylated farnesal (BF), biotinylated C_{55}-polyprenal (BP55), and biotinylated C_{80}-polyprenal (BP80), and examined their effects on *in vitro* dolichol synthesis from farnesyl diphosphate. BF and BP55 did not affect the dolichol synthesis, whereas BP80 inhibited the reduction pathway from polyprenol to dolichol, accompanied by a decrease in the entire polyprenol and dolichol synthesis. Comparison of BP80 with eighteen detergents, including Triton X-100, CHAPS, octylglucoside, deoxycholate, and Tween 80, revealed the specific effect of BP80 on the reduction pathway. On SDS–polyacrylamide gel electrophoresis, BP80 was detected in an associated form with a 50 kDa protein. These results suggest that the reduction of polyprenol to dolichol in the dolichol biosynthetic pathway proceeds with the recognition of the polyprenol chain length by a 50 kDa protein.

Key words: dolichol, *N*-linked glycoprotein biosynthesis, polyprenol, polyprenol reduction, reductase.

Since the discovery of the involvement of the long-chain polyprenyl phosphate, dolichyl phosphate, in the pathway for the assembly of the oligosaccharide chains of *N*-linked glycoproteins (*1*, *2*), a great deal of progress has been made in the understanding of the biosynthetic pathway for dolichol (*3–9*). The pathway consists of mainly two stages. The initial stage is the pathway responsible for carbon backbone synthesis of dolichol (dimethylallyl-PP → farnesyl-PP → polyprenyl-PP, **I**), and second stage is the pathway for functional group conversion (polyprenyl-PP → dolichol, **II**). The backbone synthesis (**I**) is catalyzed by farnesyl-PP synthase and polyprenyl-PP synthase. The former farnesyl-PP synthesizing pathway is identical with those of the biosynthesis of cholesterol, ubiquinone, and farnesylated proteins, and the latter pathway involves *cis*-isoprene double bond formation of polyprenyl-PP (*10*, *11*). As for the functional group conversion (**II**), several mechanisms have been proposed (*7*, *8*, *12–14*), but at present one pathway (polyprenyl-PP → polyprenyl-P → polyprenol → dolichol) is thought to be the major pathway (*13*, *14*).

Poulter and his colleagues (*15*) have purified farnesyl-PP synthase in **I** by affinity chromatography using a hydrophobic geranyl homolog ligand. Sagami *et al.* (*16*, *17*) also purified geranylgeranyl-PP synthase by similar affinity chromatography using a farnesyl homolog ligand. These results indicate that farnesyl-PP and geranylgeranyl-PP synthases have a recognition site for the hydrophobic prenyl parts of their substrates. Polyprenyl-PP synthase (farnesyl-PP → polyprenyl-PP) in **I** seems to have a similar recognition site for the hydrophobic part of its substrate. On the other hand, whether or not a series of enzymes such as polyprenyl-PP pyrophosphatase, polyprenyl-P phosphatase, and polyprenol reductase in **II** have a similar recognition site for the hydrophobic polyprenyl parts of their substrates has not been examined so far. If there is the recognition in these processes, it might be related to the carbon chain length. To understand the recognition of the hydrophobic polyprenyl part in the functional conversion process, we chemically synthesized three biotinylated polyprenyl analogs with chain lengths of C_{15}, C_{55}, and C_{80} (*18*, *19*), and examined their effects on *in vitro* dolichol synthesis.

In this paper, we describe that a biotinylated C_{80}-polyprenyl compound specifically acts as an inhibitor in the reduction pathway of polyprenol to dolichol, accompanied by association of the compound with a 50 kDa protein.

EXPERIMENTAL PROCEDURES

Materials—*Saccharomyces cerevisiae* haploid strain A364A (*MATα ade1 ade2 ura1 lys2 tyr1 his7 gal1*) used as the wild type and the #149 mutant were the same as described previously (*14*, *20*). [1-^{14}C]Isopentenyl-PP (52 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Farnesyl-PP was prepared according to the method of Davisson *et al.* (*21*). Polyprenol (C_{75}-C_{85}, P5284) and dolichol (C_{90}-C_{95}, D4511), used as standard compounds, glucose-6-phosphate dehydogenase (type VII, baker's yeast), acid phosphatase (type I, wheat germ), and protease inhibitors (leupeptin, chymostatin, antipain, pepstatin A, and aprotinin) were obtained from Sigma. Biotin-$(AC_5)_2$-hydrazide (6-(6-hydrazidohexyl)amidohexyl D-biotinamide) was

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Abbreviations: BF, biotinylated farnesal; BP, biotinylated polyprenal; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ABC, avidin biotin complex; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium chloride; PP, diphosphate.

purchased from Wako Pure Chemical Industries. Zymolyase 100T was from Seikagaku Kogyo. Silica gel 60 thin-layer and reverse-phase LKC18 thin-layer plates were purchased from Merck and Whatman, respectively. All other chemicals were of reagent grade.

Synthesis of Biotinylated Prenyl Compounds—Farnesol (C_{15}), a shorter-chain alcohol, was purchased from Aldrich. Polyprenol (C_{55}-C_{60}), a medium-chain alcohol, from silk worm feces was obtained from Takasago Perfumery Company. Polyprenol (C_{75}-C_{85}), a longer-chain alcohol, was isolated and purified as described in a previous report (*22*). Briefly, leaves of *Ginkgo biloba* were collected in November. The leaves (100 g) were dried for 1–2 days at 60°C, crushed into small pieces, and then immersed in 1,000 ml of solvent, acetone/hexane (1:1), for one week. The lipid extracts were saponified at 70°C for 2 h in 200 ml of 50% methanol containing 2 M KOH and 0.5% pyrogallol. Non-saponifiable lipids were extracted with hexane, and the solvent was evaporated off. The lipid extract was applied to a silica gel 60 column (Merck, 100 g, ϕ = 30 mm). Polyprenol was eluted with hexane/ethyl acetate (9:1), and then purified on a reverse-phase silica gel LiChroprep RP-18 column (Merck, 15 g, ϕ = 15 mm). Elution with acetone/methanol (9:1) gave polyprenol (C_{75}-C_{85}) (107.7 mg). The purity of the polyprenol was confirmed by two-plate thin-layer chromatography (*23*).

Farnesal (C_{15}), polyprenal (C_{55}-C_{60}), and polyprenal (C_{75}-C_{85}) were prepared from farnesol (C_{15}), polyprenol (C_{55}-C_{60}), and polyprenol (C_{75}-C_{85}), respectively, according to the method of Attenburrow *et al.* (*24*). Briefly, farnesol (47 mg, 210 μmol), polyprenol (C_{55}-C_{60}) (22 mg, 28 μmol), and polyprenol (C_{75}-C_{85}) (22 mg, 20 μmol) were treated with active MnO_2 (300 mg) in 5 ml chloroform at room temperature for 24 h. The mixture was centrifuged at 10,000 ×*g* for 10 min to remove the brown pellets. The chloroform extracts were dried under vacuum. Each aldehyde was quantitatively obtained.

Biotinylated prenyl compounds were synthesized using a coupling reaction between prenyl aldehyde and biotin-$(AC_6)_2$-hydrazide. Briefly, farnesal (45 mg, 200 μmol), polyprenal (C_{55}-C_{60}) (21 mg, 26 μmol), and polyprenal (C_{75}-C_{85}) (22 mg, 20 μmol) were mixed with 96 mg (200 μmol), 12 mg (26 μmol), and 9.7 mg (20 μmol) of biotin-$(AC_6)_2$ hydrazide in 2 ml of ethanol, respectively, and then stirred at room temperature for 2 h. The resulting mixture was dried, dissolved in chloroform/methanol (9:1), and then applied to a silica gel column (Merck, 30 g, ϕ = 15 mm). Elution with chloroform/methanol (9:1) gave 101 mg (74%) of biotinylated farnesal (BF), 29 mg (91%) of biotinylated polyprenal (C_{55}-C_{60}) (BP55), or 29 mg (94%) of biotinylated polyprenal (C_{75}-C_{85}) (BP80). Each biotinylated compound was obtained as a white powder (stable in water, and soluble in chloroform, ethanol and methanol). **BF**: FW = 687.03 (calcd), R_f = 0.40 [silica gel, chloroform/methanol (4:1)], ^{1}H-NMR (400 MHz, CD_3OD) δ 1.60 and 1.61 (s, 9H, *trans* CH_3), 1.68 [s, 3H, *cis* (ω)CH_3], 3.16 (m, 1H, SCH), 4.36 (b, 2H, NHCH), 5.12 (t, 2H, =CH), 5.92 (d, 1H, J = 7 Hz, N=CH), 6.03 (d, 1H, J = 8 Hz, =CHCH=N), 6.62 and 6.78 (bs, 2H, NHC=O); **BP55** (C_{55}): FW = 1,230.91 (calcd), R_f = 0.48 [silica gel, chloroform/methanol (4:1)], ^{1}H-NMR (400 MHz, $CDCl_3$) similar to BP80; **BP80** (C_{80}): FW = 1,571.48 (calcd), R_f = 0.57 [silica gel, chloroform/methanol (4:1)], ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.60 and 1.61 (s, 9H, *trans* CH_3), 1.68 (s, 39H, *cis* CH_3), 1.75 (s, 3H, CH_3C=CH), 3.16 (m, 1H, SCH), 4.36 (b, 2H, NHCH), 5.12 (t, 15H, =CH), 5.95 (d, 1H, J = 7 Hz, N=CH), 6.13 (d, 1H, J = 12 Hz, =CHCH=N), 6.62 and 6.78 (bs, 2H, NHC=O).

Analysis of the Water-Solubility of Biotinylated Compounds—In order to determine the water-solubility of BF, BP55, and BP80, a Chugaev assay was carried out according to the method of Hilderson *et al.* (*25*). The Chugaev reagent [chloroform/zinc chloride–acetic acid solution/acetylchloride (2:1:1)] was freshly prepared. The zinc chloride–acetic acid solution was prepared by heating 10 g anhydrous zinc chloride with 40 ml glacial acid for 30 min at 60°C. To BF, BP55, or BP80 (1 to 20 nmol) was added 100 μl water and the resulting solution was kept for 5 min at room temperature. The mixture was centrifuged, and the resulting water solution was extracted with 100 μl of chloroform. To the chloroform solution was added 0.5 ml of the Chugaev solution, and the resulting mixture was heated at 70°C for 30 min, cooled on ice for 5 min, and then analyzed by measurement at 388 nm.

Enzyme Preparations—Crude enzymes were prepared from yeast cells according to the method of Sagami *et al.* (*14*). Yeast cells were grown in YPD medium (2% Bacto-peptone, 1% Bacto-yeast extract, 2% glucose, and 0.003% adenine sulfate) at 23°C to the logarithmic phase, collected, washed, suspended in 100 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, and then incubated at 23°C for 5 min. The cells were collected, resuspended in a buffer containing 10 mM Tris-HCl (pH 7.5), 1.2 M sorbitol, 0.75% Bacto-yeast extract, 1.5% Bacto-peptone, 0.5% glucose, and 0.2 mg/ml Zymolyase 100T, and then incubated at 23°C for 30 min. Spheroplasted cells were collected, washed with 1.2 M sorbitol, resuspended in a preparation buffer containing 20 mM Hepes-NaOH (pH 7.5), 1 mM potassium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.6 mg/ml protease inhibitors (leupeptin, chymostatin, antipain, pepstatin A, and aprotinin), and 1 mM phenylmethylsulfonyl fluoride, and then homogenized with 30 strokes in a Teflon glass homogenizer. The homogenates were centrifuged at 1,000 ×*g* for 30 min, and the resulting supernatant was used as the crude enzymes. Protein concentrations were determined with DC protein assay reagents (Bio-Rad).

Assay Conditions and Product Analysis—The assay mixture, with a volume of 180 μl, comprising 55 mM Tris-HCl buffer (pH 8.0), 1.1 mM dithiothreitol, 111 mM potassium fluoride, and an appropriate amount of 1,000 ×*g* supernatant, was preincubated after the addition of BF, BP55, or BP80 in an ethanol solution (less than 10 μl) at 4°C for 30 min. Then, [1-^{14}C]isopentenyl diphosphate, farnesyl diphosphate, and NADPH were added to the mixture. The mixture for enzymatic reactions, with a final volume of 200 μl, comprising 50 mM Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol, 100 mM potassium fluoride, 50 μM [1-^{14}C]isopentenyl diphosphate, 40 μM farnesyl diphosphate, 1 mM NADPH, and 2.5 mg/ml 1,000 ×*g* supernatant, was incubated at 23°C for 1 h. The enzymatic products were extracted with pentane for 3 times, and then analyzed by two-plate thin-layer chromatography (*23*). Quantitative analysis of the products detected on TLC plates was performed with a Bio-image Analyzer BAS1000 (Fuji Film).

Detection of BP80 Binding Proteins—A mixture, with a volume of 50 μl, comprising 50 mM Tris-HCl buffer (pH

8.0), 1 mM dithiothreitol, 100 mM potassium fluoride, 1 mM NADPH, and 50 μg 1,000–3,000 ×*g* precipitate, was incubated after the addition of BF, BP55 or BP80 in an ethanol solution (less than 2 μl) at 23°C for 1 h. To the mixture was added sodium dodecyl sulfate to a final concentration of 0.1%. The mixture was then divided into two equal aliquots; one aliquot was subjected to SDS–polyacrylamide gel electrophoresis, the gel being stained with Coomassie Brilliant Blue, and the other was similarly electrophoresed and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The concentration of SDS in the running buffer on electrophoresis and in the blotting buffer was kept at 0.1%. The membrane was treated with 10 mM Hepes buffer [10 mM Hepes (pH 7.5), 150 mM NaCl, and 1 mM $MgCl_2$] containing 15 ng/ml avidin, 5 mU/ml biotin alkaline phosphatase, and 0.1% BSA. After 3 h at room temperature, the membrane was washed with the same buffer twice and then treated with carbonate buffer (100 mM $NaHCO_3$, 1 mM $MgCl_2$) containing 0.3 mg/ml NBT and 0.15 mg/ml BCIP (*18*).

RESULTS

In a preliminary experiment, the synthesized biotinylated compounds, as shown in Fig. 1, were found to be easily soluble in organic solvents such as chloroform, ethanol, and methanol, but less soluble in water. To determine the limitations of their water-solubilities, we analyzed the water-soluble biotinylated compounds by means of a simple colorimetric method involving the Chugaev reaction (*25*), which allows the rapid and reliable determination of isoprenoids with several double bonds in the microgram range. We first placed 1 μg of BF, BP55, or BP80 in a test tube, and then mixed it with 100 μl of water. The water-soluble compounds were extracted with chloroform and then analyzed by the method described above. As a control, the same amount (1 μg) of each of the compounds was dissolved in chloroform and analyzed similarly. The two absorption values were similar to each other, indicating that the compounds dissolved in water can be completely recovered into the chloroform phase. We next took 1 to 30 μg of each of these compounds and analyzed them in a similar way. Figure 2 summarizes the water-solubilities. BF, BP55, and BP80 exhibit water-solubilities of 9.3, 12.3, and 15.7 μg in 100 μl at 25°C, the molar absorptivities at 388 nm (ε) being 2,900, 4,600, and 5,000, respectively. These results indicate that BF, BP55, and BP80 are water-soluble at concentrations less than 140, 100, and 100 μM, respectively.

We have proposed the dolichol biosynthetic pathway (farnesyl-PP → polyprenyl-PP → polyprenyl-P → polyprenol → dolichol) in rat (*13*). This pathway was found to be localized in membranes, and polyprenol is synthesized from farnesyl-PP and isopentenyl-PP as a major product under *in vitro* assay conditions without NADPH. In the presence of NADPH, dolichol formation is observed. We have tried to establish the conversion of polyprenol into dolichol in an *in vitro* assay system of yeast as well as of rat. However, in the yeast system, exogenously added polyprenol was not converted into dolichol. We examined the effects of water-soluble BF (140 μM), BP55 (100 μM), and BP80 (100 μM) on the *in vitro* dolichol synthesis from farnesyl-PP. As shown in Fig. 3, neither BF nor BP55 inhibited the dolichol synthesis, but BP80 strongly inhibited the dolichol synthesis from polyprenol, though the polyprenol synthesis was not affected. There was no difference in the chain length distribution between polyprenol and dolichol in the presence or absence of BF, BP55, and BP80. To understand the inhibitory effect of BP80, we quantitatively analyzed the radioactive polyprenol and dolichol. As shown in Table I, the formation of polyprenol and dolichol corresponding to the total *cis*-polyprenyl products in the *in vitro* assay was reduced 85, 82, and 52% on the addition of BF, BP55, and BP80, respectively. The ratio of polyprenol and dolichol formation in the presence of BF and BP55 was almost the

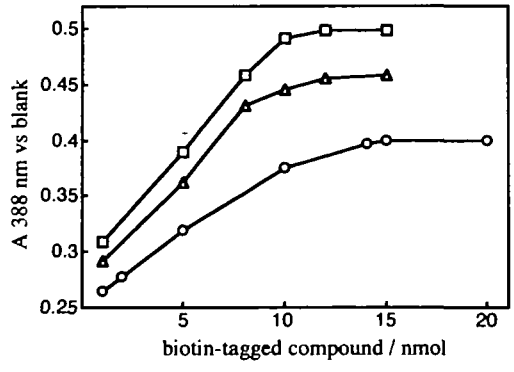

Fig. 2. **Water-solubility of BF, BP55, and BP80.** BF, BP55, or BP80 was placed in a test tube and mixed with 100 μl water. Water-soluble compounds were extracted with chloroform, and then analyzed as described under "EXPERIMENTAL PROCEDURES." BF (○); BP55 (△); BP80 (□).

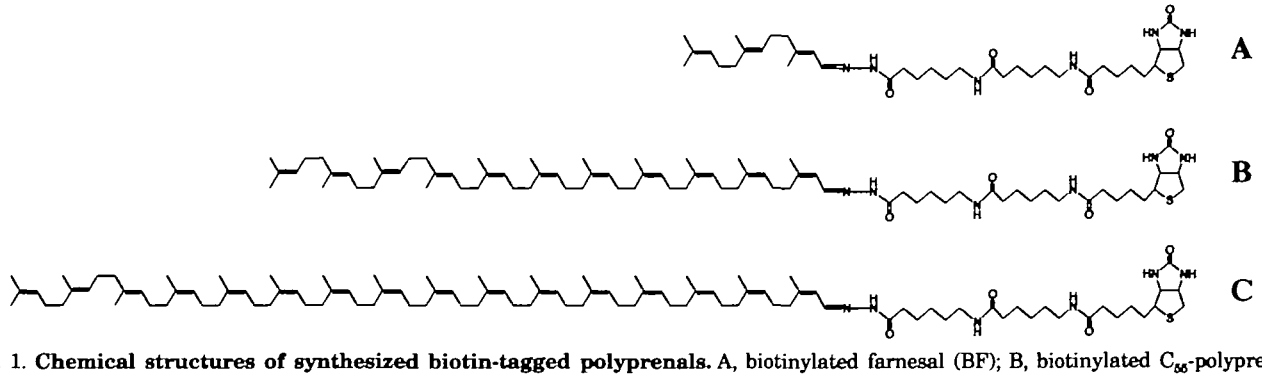

Fig. 1. **Chemical structures of synthesized biotin-tagged polyprenals.** A, biotinylated farnesal (BF); B, biotinylated C_{55}-polyprenal (BP55); C, biotinylated C_{80}-polyprenal (BP80).

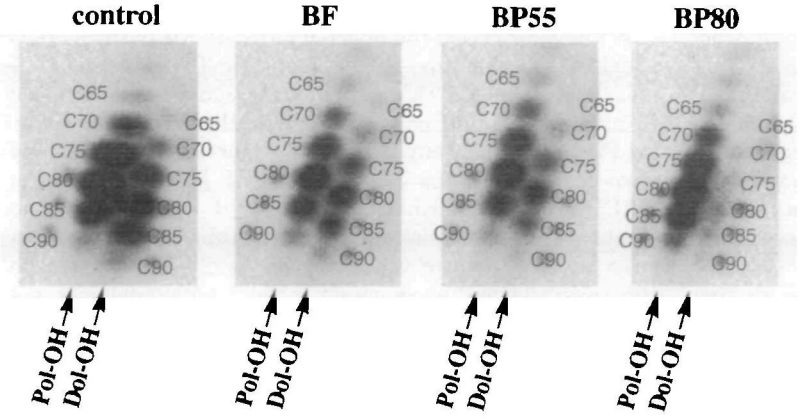

Fig. 3. **Two-plate thin-layer chromatograms of enzymatic products in the presence of BF, BP55, and BP80.** The crude enzyme was preincubated in the presence of BF (140 μM), BP55 (100 μM), or BP80 (100 μM), and then assayed as described under "EXPERIMENTAL PROCEDURES." Radioactive enzymatic products are shown. The numbers refer to the carbon chain lengths of polyprenol (Pol-OH) and dolichol (Dol-OH).

TABLE I. **Effects of BF, BP55, and BP80 on dolichol synthesis.**

	Polyprenol (A) (dpm)	Dolichol (B) (dpm)	A+B (dpm)	(%)	A:B (ratio)
Control	8,560	7,340	15,900	(100)	54:46
BF	7,240	6,330	13,600	(85)	53:47
BP55	7,340	5,790	13,100	(82)	56:44
BP80	7,040	1,210	8,250	(52)	85:15

The radioactive enzymatic products (polyprenol and dolichol) in Fig. 3 were quantitatively analyzed with a Bio-image analyzer BAS 1000.

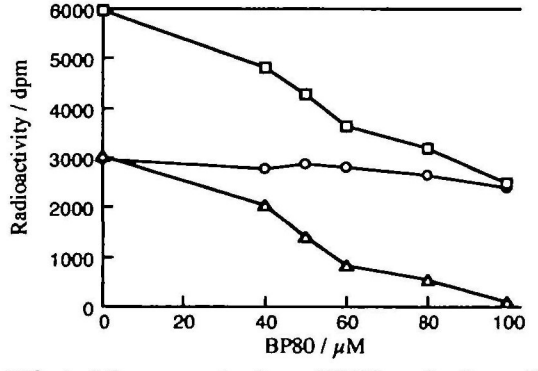

Fig. 4. **Effect of the concentration of BP80 on the formation of polyprenol and dolichol.** The enzymatic products formed in the presence of various concentrations of BP80 were analyzed as described under "EXPERIMENTAL PROCEDURES." Polyprenol (○); dolichol (△); dolichol + polyprenol (□).

same as that in the case of the control, but the ratio in the presence of BP80 was shifted toward increased polyprenol and decreased dolichol formation. These results indicate that biotinylated compounds inhibit the entire *in vitro cis*-polyisoprenoid synthesis (dolichol and polyprenol), and that BP80 among the biotinylated compounds also inhibits the reduction process from polyprenol to dolichol. To confirm this inhibitory effect, we further examined the effects of the BP80 concentration on the formation of polyprenol and dolichol. As shown in Fig. 4, the total polyprenol and dolichol formation decreased with the increase in the concentration of BP80 and the reduction process was in fact inhibited similarly. To determine whether or not the inhibition of the total polyprenol and dolichol formation by BP80

TABLE II. **Effects of BP80 and several detergents on *in vitro* dolichol synthesis.**

	Polyprenol (A) (dpm)	Dolichol (B) (dpm)	A+B (dpm)	(%)	A:B (ratio)
Control	2,410	2,030	4,440	(100)	54:46
BP8	2,020	170	2,190	(49)	92:8
Triton X-100	1,150	440	1,590	(36)	72:28
CHAPS	1,030	530	1,560	(35)	66:34
Octyl glucoside	1,520	1,000	2,520	(57)	60:40
Deoxycholate	1,080	580	1,660	(37)	65:35
Tween 80	870	440	1,310	(30)	66:34

The enzymatic products found in the presence of 100 μM BP80 or one of several detergents was analyzed as described under "EXPERIMENTAL PROCEDURES." The radioactive enzymatic products (dolichol and polyprenol) were quantitatively analyzed with a Bio-image analyzer BAS 1000.

is accompanied by increased formation of polyprenyl mono- and diphosphate, we examined the effects of BP80 on polyprenyl mono- and diphosphate synthesis. No increased formation of these minor intermediate products was observed in the presence of BP80 (data not shown).

In a previous report, we described that several detergents, such as Triton X-100, CHAPS, octylglucoside, and deoxycholate, completely inhibited the dolichol synthesis at their critical micellar concentrations (*14*). Considering this detergent inhibitory effect, we next compared the inhibitory effect of BP80 with those of several detergents at 100 μM. As shown in Table II, all the detergents examined showed similar inhibitory effects to BP80 on the entire *cis*-polyisoprenoid formation (polyprenol and dolichol), octylglucoside having less effect than BP80. The inhibitory effect on the reduction process from polyprenol to dolichol was in the order of BP80 > Triton X-100 > CHAPS = Tween 80 > deoxycholate > octylglucoside. These results indicate that BP80 has the ability to inhibit the entire *cis*-polyisoprenoid synthesis similar to detergents, but also the ability to more specifically inhibit the reduction process than detergents. Further, we also examined the effects of other detergents, such as CHAPSO, BIGCHAP, deoxy-BIGCHAP, *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-thioglucoside, *n*-dodecyl-β-D-maltoside, MEGA-8, MEGA-9, MEGA-10, sucrose monocaprate, sucrose monolaurate, cholate, and digitonin. However, none of the detergents examined inhibited the reduction process more specifically than BP80 (data not shown).

Having established that BP80 specifically inhibits the polyprenol reduction process, we further examined whether

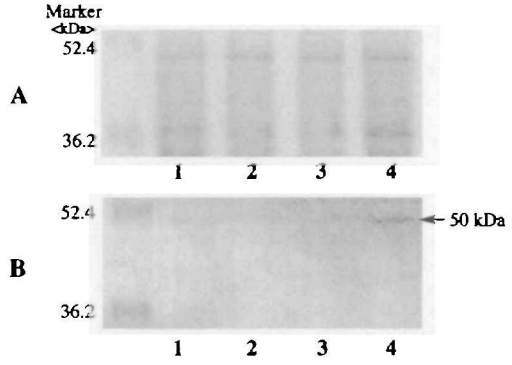

Fig. 5. **SDS-PAGE of the 1,000–3,000 ×*g* precipitate fraction preincubated with BF, BP55, and BP80.** An active 1,000–3,000 ×*g* precipitate fraction was mixed with BP80 or BF, and then incubated at 23°C and analyzed as described under "EXPERIMENTAL PROCEDURES." The transferred protein containing biotin-tagged polyprenal was treated with avidin and biotin alkaline phosphatase, followed by detection with NBT and BCIP (Panel B). Lane 1, precipitate fraction alone; lane 2, precipitate fraction + BF; lane 3, precipitate fraction + BP55; lane 4, precipitate fraction + BP80.

or not the hydrophobic interaction of BP80 is strong enough for the detection of BP80-protein complexes on SDS-PAGE. Surprisingly, a faint but discriminable band (*ca.* 50 kDa) was only detected in the presence of BP80 (Fig. 5, panel B, lane 4). On the other hand, this band was not detected in the case of BF or BP55. We also used the 100,000 ×*g* supernatant fraction and observed a band (*ca.* 15 kDa) in the case of presence of BP80. However, this band could also be detected without BP80. These results imply that a protein corresponding to 50 kDa specifically recognizes the hydrophobic polyprenyl part of BP80.

DISCUSSION

The original aim of this study was to determine whether or not, in the biosynthetic pathway for a series of dolichols and sugar-linked dolichols, the long hydrophobic parts of these compounds are recognized. In a trial to establish the dolichol biosynthetic pathway, exogenously added polyprenol was in fact converted to dolichol with a rat microsomal fraction in the presence of NADPH, though the conversion was extremely low (*13*). But in the case of a yeast membrane fraction, similar conversion was not observed. We assumed the occurrence of a putative polyprenol binding protein in the reduction process. Since polyprenol is a neutral compound, it would be incorporated inside the membranes without the putative binding protein. Because the reduction process with an attack by the hydride of NADPH is thought to proceed near the cytoplasmic side of the membrane, the α-isoprene part of polyprenol must be located not inside but outside the membrane, and the hydrophobic part of polyprenol must be located inside the membrane in an associated form with the putative binding protein. We mainly arranged the dolichol biosynthetic pathway in three parts: the carbon backbone synthetic pathway (dimethylallyl-PP → polyprenyl-PP, **I**), the functional group conversion pathway (polyprenyl-PP → dolichyl-P, **II**), and the sugar carrier synthetic pathway (dolichyl-P → oligosaccharyl-PP-dolichol, **III**). In the present study, we focused on pathways **I** and **II** because we have established an *in vitro* assay system including pathways **I** and **II**. Considering the hydrophobic chain lengths of the compounds in **I** and **II**, we chemically synthesized three kinds of biotin-tagged polyprenal compounds (BF, BP55, and BP80), and examined their effects on the *in vitro* dolichol assay system of yeast. Only BP80 specifically inhibited the polyprenol reduction pathway and was detected in a form associated with a 50 kDa protein. Since this 50 kDa protein is thought to associate with only the hydrophobic prenyl parts of biotin-tagged compounds, the protein might also associate with BF or BP55. However, the 50 kDa protein was not detected in the presence of BF or BP55. This means that only the unusually long hydrophobic part of polyprenol (C_{80}) can be at least recognized in dolichol synthesis from polyprenol in pathway **II**. As for enzymes in pathway **III**, Shibaev *et al.* (*26*) recently reported that derivatives of dolichyl phosphate (C_{70}-C_{85}) with 2-aminopyridine or 1-aminonaphtalene groups at the ω-end of the carbon chain served as substrates for recombinant yeast mannosylphosphoryldolichol synthase. Whether or not enzymes other than mannosylphosphoryldolichol synthase in pathway **III** recognize the hydrophobic chain length in the catalytic reaction was not studied by them.

Szkopinska *et al.* (*27*) reported that overexpression of farnesyl diphosphate synthase in an *erg* 9 mutant of yeast *Saccharomyces cerevisiae*, defective in squalene synthase activity, resulted in a 100-fold increase in the amount of polyprenol in comparison with dolichol in the wild type. Interestingly, in addition to the typical yeast dolichol (C_{70}-C_{80}), the chain length of polyprenol ranged up to C_{135}. These results indicate that the normal chain length polyprenol (C_{70}-C_{80}), as *de novo* intermediate, was predominantly converted into dolichol, but polyprenol with longer chain length was not accepted as substrate by polyprenol reductase, supporting the chain length specificity in the reduction process described in the present study.

Recently, Moody *et al.* (*28*) described that the CD1c-restricted mycobacteria-specific T cell line could recognize mannosyl-β1-phosphodolichol. They also examined the effects of chain length and α-double bond for isoprenoid residue of the antigen. As a result, the C_{35} dolichol type was recognized more strongly than the C_{55} dolichol type, and the C_{95} dolichol type showed weak responses. In the case of the polyprenol type, no response was observed though the chain length was C_{35}. These results indicate that the CD1c-restricted T cell line exhibits fine specificity for the hydrophobic prenyl chain, and recognizes the chain length as well as the α-saturated isoprene bond of mannosylphosphoryldolichol. The carbon chain length of mannosylphosphoryldolichol, as an immunological antigen, and of polyprenol, as a dolichol biosynthetic intermediate, seem to be recognized with fine specificity in spite of their unusually long hydrophobic parts.

Ericsson *et al.* (*29*) have reported that the activity of polyprenyl diphosphate synthase bound to microsomes was stimulated by the addition of activating factors in a 100,000 ×*g* soluble fraction, one of which was identified as sterol-binding protein 2. Sagami *et al.* (*13*) have also found that the microsomal polyprenol reductase activity was stimulated 9-fold by the addition of a 100,000 ×*g* soluble fraction of a rat liver homogenate. In the case of yeast, the microsomal reductase activity was not stimulated by the addition of the 100,000 ×*g* soluble fraction (data not shown). It is

possible to assume that the putative activating factor for the reductase activity of yeast is associated with microsomes, constituting a complex with the apo-reductase. BP80 might interact with the putative activator rather than with the apo-reductase, and the putative activator might be the 50 kDa protein found in the present study.

It has been reported that Chinese hamster ovary cells of the Lec 9 recessive complementation group contain a lower amount of dolichol, and the mutants biosynthesize underglycosylated glycoprotein (*30*, *31*). One of the mutants, F2A8 cells, accumulated 10 times more polyprenol than the wild type, showing that F2A8 cells completely lack polyprenol reductase (*32*). Ohkura *et al.* (*33*) have reported that the carbohydrate-deficient glycoprotein syndrome type I, which is a congenital disorder involving the underglycosylation of *N*-glycosylated glycoproteins, involves the re-duced synthesis of dolichol from polyprenol. These results suggest that a deficiency of polyprenol reduction, even though partial, is the primary cause of underglycosylated glycoproteins. To better understand the mechanism of this reduction in the reduction of polyprenol to dolichol, the occurrence of a 50 kDa protein, which exhibits higher affinity to the hydrophobic part of polyprenol, should be considered. Further experiments are necessary to clarify the recognition of the hydrophobic parts of polyprenol, dolichol, and sugar-linked dolichol by other enzymes in pathways **II** and **III**.

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