Enzymatic Characterization of Human α1,3-Fucosyltransferase Fuc-TVII Synthesized in a B Cell Lymphoma Cell Line*

(Received for publication, July 29, 1997, and in revised form, October 2, 1997)

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The human α1,3-fucosyltransferase, Fuc-TVII, a key enzyme in the biosynthesis of selectin ligands, was expressed as a soluble protein-A chimeric form in a human B cell lymphoma cell line, Namalwa KJM-1, and purified using IgG-Sepharose. The enzymatic properties of recombinant soluble Fuc-TVII were then examined. Its enzyme activity was highest at pH 7.5, and the presence of 25 mM Mn²⁺ was required for full activity. Fuc-TVII exhibits an acceptor specificity restricted to α2,3-sialylated type 2 oligosaccharides, and the apparent Km values for α2,3-sialyl lacto-N-neotetraose and GDP-fucose were 3.08 mM and 16.4 μM, respectively. The inhibitory effects of various nucleotides on the activity of Fuc-TVII reflected its donor specificity for the nucleotide portion of GDP. Fuc-TVII was demonstrated to be useful for the synthesis of a sialyl Lewis x hexasaccharide from lacto-N-neotetraose in combination with an α2,3-sialyltransferase, ST3Gal IV. Polyethylene glycols enhanced the thermal stability of Fuc-TVII, leading to increased formation of the reaction product.

Cell surface glycoproteins and glycolipids containing α1,3-fucosylated lactosaminoglycans play important roles in cell-cell interaction and cell migration in connection with physiological and pathological processes such as embryogenesis, cancer metastasis, lymphocyte trafficking, and immune responses (1, 2). In particular, the sialyl Lewis x (sLex; NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc) oligosaccharide structure and related structures have evoked considerable interest because they were demonstrated to serve as ligands for the three known selectins (E-, P-, and L-selectins), which are cell adhesion molecules involved in the recruitment of leukocytes to lymphoid tissues and the sites of inflammation (2–6).

The biosynthesis of α1,3-fucosylated lactosaminoglycans requires the actions of several glycosyltransferases. Among them, an α1,3-fucosyltransferase (Fuc-T) is considered to be the key enzyme, which catalyzes the transfer of fucose from GDP-fucose to N-acetylglucosamine via an α1,3-linkage. The expression-cloning approach led to the cloning of the first human α1,3-Fuc-T, Fuc-TIII (7). Subsequently, low stringency hybridization-cloning and expression-cloning approaches revealed the cDNAs for three additional members, Fuc-TIV, Fuc-TV, and Fuc-TVII (8–13). Detailed analyses have shown that Fuc-TIII, Fuc-TV, and Fuc-TVII correspond to Lewis-type, myeloid-type, and plasma-type enzymes, respectively (7–10, 14). Biochemical analyses involving enzyme preparations extracted from human tissues and/or the respective recombinant proteins revealed their acceptor specificities, pH optima, kinetic properties, cation requirements, and N-ethylmaleimide (NEM) sensitivities (15–20).

We (21) and Natsuka et al. (22) previously cloned a novel human α1,3-Fuc-T, Fuc-TVII, expressed in myeloid lineage cells that had never been characterized biochemically. We presented evidence of its involvement in the biosynthesis of an E-selectin ligand. Recent studies have also demonstrated its involvement in the biosynthesis of E-, P-, and L-selectin ligands (23, 24). As expected, Fuc-TVII-deficient mice were shown to exhibit a leukocyte adhesion deficiency that was characterized by both the absence of leukocyte E- and P-selectin ligand activity, and a deficiency of L-selectin ligand activity in high endothelial venules (23). Therefore, the inhibition of Fuc-TVII activity should suppress the expression of selectin ligands thereby reducing selectin-mediated leukocyte adhesion to endothelial cells. Selective inhibitors of Fuc-TVII are expected to be potential therapeutics for the treatment of inflammatory diseases.

The enzymatic synthesis of oligosaccharides using specific glycosyltransferases is an efficient approach for preparing them on a large scale. α1,3-Fuc-Ts will be valuable tools for the in vitro synthesis of sLeα oligosaccharides that may be used as selectin-mediated adhesion blockers. Indeed, Fuc-TIII was shown to be useful for the enzymatic synthesis of a sLeα tetrasaccharide in combination with an α2,3-sialyltransferase, ST3Gal III (25). We (21) and Natsuka et al. (22) previously revealed that Fuc-TVII exhibits an acceptor specificity restricted to α2,3-sialylated type 2 oligosaccharides. This rigid acceptor specificity of Fuc-TVII suggests that it may be suitable for the in vitro large-scale synthesis of sLeα oligosaccharides.

Whereas the biological role of Fuc-TVII has been extensively investigated (21–24), its enzymatic properties have been poorly characterized except for its acceptor specificity. Further characterization of Fuc-TVII will be useful for understanding the difference in enzymatic properties between α1,3-Fuc-Ts. Elucidation of its properties in detail will advance the discovery of its inhibitors and facilitate its use for the large-scale synthesis of sLeα oligosaccharides. For determination of the enzymatic properties of Fuc-TVII in this study, we used its soluble recombinant protein A chimeric form produced by Namalwa KJM-1,
Characterization of Fuc-TVII

EXPERIMENTAL PROCEDURES

Preparation of Soluble Glycosyltransferases Fused with Protein A—A fragment encoding the secretable Fuc-TVII protein fused with protein A was excised from pMaOa-FT7 (21) and then cloned into a mammalian expression vector, pAGE248 (21) carrying the dihydrofolate reductase gene, which gave plasmid pAGE248-AFT7. After transfection of Namalwa KJM-1 cells with this plasmid, a clone, F7C4, producing ~0.6 µg/ml of the soluble protein Fuc-TVII fusion protein was obtained using the dihydrofolate reductase gene coamplification method described by Miyaji et al. (26). This clone was cultured in RPMI 1640 medium containing 10% IgG-free fetal calf serum (GF21; Wako Pure Chemicals), and then the secreted Fuc-TVII protein in the conditioned medium was applied to an IgG-Sepharose column (Pharmacia Biotech Inc.). The Fuc-TVII protein was eluted from the column with 0.5 M acetic acid (pH 3.5), immediately neutralized with 2 M Tris, and then desalted by gel filtration on a Sephadex G-25 M column (Pharmacia) equilibrated with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.0). The protein concentration was determined with a Bio-Rad protein kit based on the method of Bradford (27) using bovine serum albumin as a standard. The purity of the Fuc-TVII protein was determined by electrophoresis on a 10% SDS-polyacrylamide gel (PAGE NLPU-10L; Atto Co., Ltd.). The prepared enzyme was detected as 50–55-kDa bands on SDS-polyacrylamide gel electrophoresis, and its purity was ~70%.

Likewise, a fragment encoding a secretable protein A fusion protein of an α2,3-sialyltransferase, ST3Gal IV, was excised from pMaOa-ST4 (28) and cloned into pAGE248 to generate plasmid pAGE248-AST4. After transfection of Namalwa KJM-1 cells with this plasmid, a clone, S4C9, producing ~11 µg/ml of the fusion protein was obtained by the same procedure as described above. The protein A-fused Fuc-TVII protein was synthesized in Namalwa KJM-1 cells transfected stably with plasmid pMaOa-FT8 (21). The recombinant enzyme was purified by the same procedure as described above and then stored at ~80°C in 50 mM MOPS (pH 7.0).

Fuc-T Assay—Standard Fuc-T assays were performed in a total volume of 30 µl containing 100 mM cacodylate buffer (pH 7.5), 25 mM MnCl2, 0.05 mM GDP-fucose, and 0.025 mM pyridylaminated α2,3-sialyl lacto-N-neotetraose (α2,3-sialyl LNNt; NeuAcα2-3Galβ1–4GlcNAcβ1–3Galβ1–4Glc), and one of the purified enzymes (1.0 mg/ml) in the reaction mixture up to 50 ng/ml (data not shown). To examine the effect of the concentration of PEG on the enzyme activity, different concentrations of PEG2000 were added to the reaction mixture at 20% (w/v). After centrifugation, the reaction mixture was added to the mixture. After incubation at 37°C for 2 h, the reaction was stopped by boiling for 5 min. The protein A-fused Fuc-TVII enzyme was purified by the same procedure as described above and then stored at ~80°C in 50 mM MOPS (pH 7.0).

The acceptor specificity of Fuc-TVII was determined using the following pyridylaminated oligosaccharides; LNNt, α2,3-sialyl LNNt, α2,6-sialyl LNNt, lacto-N-tetraose (Galβ1–3GalCNAcβ1–3Galβ1–4Glc), α2,3-sialyl lacto-N-tetraose, lacto-N-fucopentaose I (Fucα1–3Galβ1–3GalCNAcβ1–3Galβ1–4Glc), lacto-N-fucopentaose V (NeuAcα2–3Galβ1–3GalCNAcβ1–3Galβ1–4(Fucα1–3Glc), and α2,3-sialyl lacto-N-fucopentaose V.

Inhibition of Fuc-TVII by Nucleotides—The enzyme activity was measured in the reaction mixture containing different concentrations of GDP-fucose (25, 50, and 100 µM) that was mixed with various nucleotides or guanosine at final concentrations of 0.12–30 mM. The K<sub>i</sub> value for each nucleotide or guanosine was calculated from Dixon plots.

lLe<sup>+</sup> Hexasaccharide Synthesis with Fuc-TVII and ST3Gal IV—3 nmol of LNNt was reacted with either Fuc-TVII or Fuc-TV in combination with ST3Gal IV in a total volume of 60 µl containing 100 mM cacodylate buffer (pH 7.0), 25 mM MnCl2, 0.5 mM CMP-N-acetylneuraminic acid, and 0.5 mM GDP-fucose. After incubation at 27°C for 24 h, the reaction products were analyzed by HPLC. The structure of the reaction product, a lLe<sup>+</sup> hexasaccharide, was confirmed by spectroscopic analyses with a Bruker AM-500 nuclear magnetic resonance spectrometer and a JEOl JMS VX/200 110A mass spectrometer.

Thermal Stability of Fuc-TVII—To examine the effect of PEG 2000 on the thermal stability of Fuc-TVII, the enzyme was incubated in 100 mM cacodylate buffer (pH 7.5) with or without 36% PEG 2000 at 4, 15, 27, 37, 45, and 50°C for 1 h and followed by measurement of its residual activity at 37°C for 2 h.

RESULTS

Preparation of Fuc-TVII—The recombinant Fuc-TVII enzyme was expressed as a soluble protein A fusion protein in Namalwa KJM-1 cells that had been transfected stably with plasmid PAGE248-AFT-7. This protein A-fused Fuc-TVII enzyme was purified from the conditioned medium using an IgG-Sepharose column and then used for further analyses.

Optimal Reaction Conditions—To determine the optimal pH for the Fuc-TVII-catalyzed reaction, the enzyme activity was measured at various pH levels in five different buffers (shown in Fig. 1) that contained 25 mM MnCl<sub>2</sub>, 0.05 mM GDP-fucose, and 0.025 mM pyridylaminated α2,3-sialyl LNNt. The highest activity was obtained around pH 7.5, as adjusted with a cacodylate buffer (Fig. 1).

Divalent cations, especially Mn<sup>2+</sup>, were reported to activate α1,3-Fuc-Ts (15, 20). We also examined the effects of divalent cations on the enzyme activity of Fuc-TVII. Among the cations tested (Mn<sup>2+</sup>, Ca<sup>2+</sup>, Cd<sup>2+</sup>, and Mg<sup>2+</sup>), the activity was most enhanced in the presence of Mn<sup>2+</sup> (Fig. 2). The activity at the optimal Mn<sup>2+</sup> concentration of 25 mM was 12-fold higher than that with no divalent cation. Manganese ions, Ca<sup>2+</sup>, and Mg<sup>2+</sup> stimulated the enzyme activity in the concentration range of 0.25–100 mM, whereas Co<sup>2+</sup> activated it in a narrower concentration range (Fig. 2). To examine the effect of NaCl on the enzyme activity, the reaction mixture was incubated in the presence of increasing concentrations of NaCl up to 2 M, followed by measurement of the activity. NaCl was found to have an inhibitory effect on the activity and to abolish it almost completely at the concentration of 1 M and higher (data not shown).

The linearity of the enzyme reaction was examined as a function of the protein concentration and the incubation period. The enzyme activity increased linearly with Fuc-TVII protein concentration in the reaction mixture up to 50 ng/ml (data not shown). The optimal reaction temperature was ~37°C when the amount of the reaction product was measured after incubation at various temperatures for 2 h (Fig. 3). At 37°C, the enzyme reaction proceeded almost linearly in the incubation period range up to 1 h. On Fuc-TVII-catalyzed reaction for 24 h, the reaction product was accumulated at a higher level at 27°C rather than at 37°C (data not shown).

Acceptor Specificity and Kinetic Studies—Fuc-TVII was shown to have an acceptor specificity restricted to α2,3-sia-
lylated type 2 oligosaccharides (21, 22). This acceptor specificity was confirmed by assays involving the purified enzyme and various acceptor oligosaccharides including α2,6-sialyl LNnT, which had never been examined for availability as acceptor substrates (data not shown). The enzyme activity of Fuc-TVII was measured as a function of varying concentrations of α2,3-sialyl LNnT, from 0.16 to 10 mM, with a fixed GDP-fucose concentration of 1.0 mM, and the apparent $K_m$ value for α2,3-sialyl LNnT was determined from Lineweaver-Burk plots to be 3.08 mM (Fig. 4A). From the plots obtained in the GDP-fucose concentration range of 0.002 to 0.3 mM with a constant α2,3-sialyl LNnT concentration of 0.25 mM, the apparent $K_m$ value for GDP-fucose was calculated to be 16.4 μM (Fig. 4B).

**Inhibition by Nucleotides**—The inhibitory effects of various nucleotides on the enzyme activity were examined to determine the specificity of Fuc-TVII for the nucleotide portion of a donor substrate. GDP was the most potent inhibitor among them (Table I), consistent with the availability of GDP-fucose as a donor. The other nucleotide diphosphates listed in Table I were much weaker inhibitors, reflecting the specificity of Fuc-TVII for the nucleoside portion. Whereas GMP and GTP exhibited considerable inhibitory effects, guanosine scarcely inhibited the enzyme activity, suggesting the requirement of a phosphate moiety for the recognition of a donor substrate by Fuc-TVII.

**Effects of NEM Treatment**—Human α1,3-Fuc-Ts have been classified as to sensitivity to NEM as well as tissue distribution and acceptor specificity (15–20). The NEM sensitivity of Fuc-TIII and Fuc-TV was shown to be due to irreversible modification by NEM of a Cys residue involved in GDP-fucose binding (29). In contrast, Fuc-TIV was insensitive to NEM, which was demonstrated to result from the presence of a Ser residue at the corresponding position (29). Fuc-TVII has been assumed to be NEM-insensitive because it contains a Thr residue at the equivalent position (21, 22). To assess the NEM sensitivity of Fuc-TVII, the reaction mixture was mixed with NEM at a final concentration of 0.12 to 30 μM and followed by preincuba-

### Table I

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$K_i$ μM</th>
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<tr>
<td>GTP</td>
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<tr>
<td>GDP</td>
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<tr>
<td>GMP</td>
<td>0.094</td>
</tr>
<tr>
<td>Guanosine</td>
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<tr>
<td>Inosine diphosphate</td>
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</tr>
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<tr>
<td>CDP</td>
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<td>UDP</td>
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tion at 4 °C for 1 h. As expected, measurement of the remaining enzyme activity revealed that it was hardly inactivated by NEM (data not shown).

**sLex Hexasaccharide Synthesis with Fuc-TVII in Combination with ST3Gal IV**—As mentioned above, α1,3-Fuc-Ts should be useful for the large-scale synthesis of sLea oligosaccharides. Fuc-TVII has an acceptor specificity restricted to α2,3-sialylated type 2 oligosaccharide, raising the possibility that when Fuc-TVII is used for such synthesis in combination with an α2,3-sialyltransferase, less by-product will be formed compared with other cloned α1,3-Fuc-Ts. To prove this possibility in a small scale synthesis, we have examined the conversion of LNNt into sLea hexasaccharide with the combination of Fuc-TVII and an α2,3-sialyltransferase, ST3Gal IV, that utilizes type 2 oligosaccharides more preferentially than type 1 oligosaccharides (30). Our independent studies on ST3Gal IV have revealed that the optimal buffer for its reaction is 100 mM MOPS buffer (pH 7.0) and that its enzyme activity is not affected by the presence of 25 mM divalent cations (data not shown). A starting substrate, LNNt, was incubated with Fuc-TVII and ST3Gal IV for 24 h under reaction conditions suitable for both enzymes, that is, at 27 °C in a reaction mixture comprising 100 mM MOPS buffer (pH 7.0), 25 mM MnCl₂, 0.5 mM CMP-N-acetylneuraminic acid, and 0.5 mM GDP-fucose, resulting in the accumulation of a sLea hexasaccharide in a yield of >90% without any detectable by-product. For comparison, we also performed the sLea hexasaccharide synthesis with Fuc-TVII instead of Fuc-TVII. When LNNt was incubated with both Fuc-TVII and ST3Gal IV, a Lewis x pentasaccharide, which is an undesirable by-product, was formed in a yield of ~20%, leading to a decreased yield (78%) of sLea hexasaccharide.

**Effect of PEG**—The stabilization of α1,3-Fuc-Ts in the synthesis of sLea oligosaccharides is an important issue. Since PEG was reported to stabilize some enzymes resulting in increased yields of their reaction products (31–33), we examined the effect of PEG on the Fuc-TVII reaction. After incubating α2,3-sialyl LNNt with Fuc-TVII at 37 °C for 2 h in a reaction mixture containing PEG of different sizes at a concentration of 20% (w/v), the amount of the sLea hexasaccharide formed increased significantly with any size of PEG added (Fig. 5A). The maximal increase (~70%) in its amount was observed in the presence of PEG 2000.

Previous studies revealed that the addition of PEG leads to an increase in the thermal stability of some enzymes (31–33). Therefore, the effect of PEG 2000 on the thermal sensitivity of Fuc-TVII was examined by preincubating at different temperatures for 1 h with or without PEG 2000, followed by measurement of its residual activity at 37 °C. In the absence of PEG 2000, Fuc-TVII was stable in the range of 4–27 °C, and >50% of its activity still remained after 1 h of incubation at 37 °C, but its activity was hardly detected at 45 °C and higher (Fig. 5B). On the other hand, in the presence of PEG 2000, the thermal stability of Fuc-TVII was enhanced, and ~55% of the enzyme activity remained after 1 h of incubation even at 50 °C (Fig. 5B). On reaction at 37 °C for 2 h in the presence of increasing concentrations of PEG up to 36%, the optimal concentration of PEG was determined to be 36%, which was almost the upper limit for its solubility (Fig. 5C).

**DISCUSSION**

In this study, the human B cell lymphoma Namalwa KJM-1 cell line was chosen as a host for the production of recombinant Fuc-TVII because a transfected gene linked to the long terminal repeat promoter of the Moloney murine leukemia virus should be efficiently expressed in this cell line (28). The COOH-terminal catalytic domain of Fuc-TVII fused with protein A was expressed as a secretable protein in Namalwa KJM-1 cells and then purified from the conditioned medium on an IgG-Sepharose column.

This purified recombinant Fuc-TVII enzyme was analyzed to determine its optimal reaction conditions. Its optimal pH was
shown to be ~7.5 in 100 mM cacodylate buffer. A previous study on the mechanism for the fucosyl transfer by Fuc-TV (20) indicated that divalent cations such as Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Co$^{2+}$, capable of adopting an octahedral geometry, formed a complex with the pyrophosphate moiety in GDP-fucose, and this accelerated its hydrolysis reaction as an electrophilic catalyst. (20). Like those of other cloned α,1,3-Fuc-Ts, the enzymatic activity of Fuc-TVII was stimulated by divalent cations (Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Co$^{2+}$), among which Mn$^{2+}$ was the most effective. Co$^{2+}$ activated the enzyme in a relatively narrow concentration range and rather exhibited an inhibitory effect on the reaction at 50 mM and higher, the reason for which remains unknown. The optimal reaction temperature for 2 h of incubation was ~37 °C, whereas after 24 h of incubation the reaction product was accumulated at a higher level at 27 °C than at 37 °C. The latter result presumably reflects thermal inactivation of Fuc-TVII at 37 °C, because prolonged incubation of it at 37 °C and higher was found to cause a loss of its activity.

The nucleotide inhibition study on Fuc-TVII revealed that the Kᵢ values of GDP and guanosine were 0.035 and >20 mM, respectively, i.e. nearly consistent with their inhibitory potencies as to Fuc-TV reported previously (20). On the other hand, GTP was a weaker inhibitor for Fuc-TVII (Kᵢ = 0.17 mM) than Fuc-TV (0.03 mM), whereas GMP was a stronger one for Fuc-TVII (Kᵢ = 0.094 mM) than Fuc-TV (Kᵢ = 0.7 mM). Furthermore, the Kᵢ values of inosine diphosphate, ADP, and GDP for Fuc-TVII (0.77, 7.3, and 11 mM, respectively) were different from those for Fuc-TV (0.069, 0.58, and 3.5 mM, respectively). These differences in nucleotide inhibitory potency between Fuc-TVII and Fuc-TV may be due to their subtle differences in the structures of their GDP-binding domains. Sequence analyses of five cloned human α,1,3-Fuc-Ts revealed that Fuc-TVII comprises a unique class of the human α,1-fucosyltransferase family exhibiting 42–43% homology with each of the three chromosome 19p-encoded α,1,3-Fuc-Ts (Fuc-TIII, Fuc-TV, and Fuc-TVII) and 47% with Fuc-TIV (21, 22). Since Fuc-TVII has been demonstrated to be involved in the biosynthesis of selectin ligands (21, 23, 24), Fuc-TVII-selective inhibitors should have therapeutic potential as anti-inflammatory drugs. Collectively, this is probably due to sialylation of the by-product by ST3Gal IV because this enzyme, albeit less efficiently, can convert a Lex oligosaccharide into a sLex oligosaccharide (28). Ichikawa et al. (25) reported the development of a sLex oligosaccharide synthesis system using ST3Gal III and Fuc-TIII. The system shown in this study may have an advantage over their system in detecting no by-product even by simultaneous addition of an α,2,3-sialyltransferase and an α,1,3-Fuc-T. They also developed an in situ regeneration system of the donor substrates sugar nucleotides to avoid product inhibition by the released nucleotide phosphates to reduce the input amounts of expensive sugar nucleotides. Therefore, the combination of our synthesis system with the in situ regeneration system would be an attractive approach for the large-scale production of sLex oligosaccharides. In this study, glycosyltransferases produced in B cell lymphoma cells were used for the small-scale synthesis of sLex oligosaccharides. For the preparation of glycosyltransferases used for the large-scale oligosaccharide production, however, the expression system using yeast (34) or fungi (20) is more appropriate than the mammalian-cell expression system because the former should yield active glycosyltransferases at much lower cost than the latter.

We have shown that PEG species of various sizes stabilize the Fuc-TVII reaction at 37 °C and higher. This is the first demonstration of enhancement of the thermal stability of a mammalian glycosyltransferase by PEG. We have examined the effect of PEG on a homologous glycosyltransferase, Fuc-TV, but no PEG species examined for Fuc-TVII activated Fuc-TV (data not shown). Thus, the effectiveness of PEG addition for the enzymatic reactions of other mammalian glycosyltransferases cannot be simply predicted. PEG addition will be useful for the synthesis of sLex oligosaccharides, especially when using Fuc-TVII.

Acknowledgments—We are grateful to Dr. Mayumi Yoshida for the nuclear magnetic resonance measurements and Yumiko Aotani for the mass spectrometric analysis. We also thank Kazumi Hasegawa, Miho Nagata, and Yukiko Kodama for their expert assistance.

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doi: 10.1074/jbc.272.51.31992

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