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MUCIN BIOSYNTHESIS. ENZYMIC PROPERTIES OF HUMAN-TRACHEAL EPITHELIAL GDP-L-FUCOSE:β-D-GALACTOSIDE α-(1→2)-L-FUCOSYLTRANSFERASE*

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ABSTRACT

The human-tracheal, epithelial α-(1→2)-L-fucosyltransferase that transfers L-fucose from GDP-L-fucose to an acceptor containing a β-D-galactopyranosyl group at the nonreducing terminal was characterized. Optimal enzyme activity was obtained at pH 6.5. 20–30mM $MnCl_2$ (or $CaCl_2$), and 0.05% Triton X-100 or 0.5% Tween 20. Mg^{2+} and Ba^{2+} ions moderately enhanced the enzyme activity, whereas Fe^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} ions were inhibitory. The enzyme activity was inhibited by *N*-ethylmaleimide and nucleotides of guanine, inosine, xanthine, and uridine. However, ATP and dithiothreitol did not affect the enzyme activity. The apparent Michaelis constant for GDP-L-fucose, freezing point-depressing glycoproteins (expressed as Gal→GalNAc→Thr), and phenyl β-D-galactopyranoside was 0.29, 5.70, and 25.4mM, respectively. Under alkali–borohydride conditions (0.05M NaOH–M $NaBH_4$, 45°, 20 h), an L-[^{14}C]fucosyltrisaccharide was released from the product obtained by use of freezing point-depressing glycoprotein as the acceptor. The α-L anomeric configuration of the fucoside was determined by the release of L-[^{14}C]fucose from the purified trisaccharide by *Turbo cornutus* α-L-fucosidase. The (1→2) linkage of the L-fucosyl group to the D-galactosyl residue was established by methylation technique (m.s.–g.l.c.). The present enzyme has properties similar to those of the human milk α-(1→2)-L-fucosyltransferase which is encoded by a secretor gene.

INTRODUCTION

The structures at the nonreducing terminus of the carbohydrate chains of some mucins possess blood groups A, B, H, Le^a, Le^b, and influenza virus-binding

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activities[2,3]. These structures are determined by specific glycosyltransferases[4–6]. Baker *et al.*[7,8] have reported that the activities of glycosyltransferases are enhanced in the tracheal epithelia of animals[7] and human subjects[8] in hypersecretory state. In addition, patients suffering from cystic fibrosis secrete more highly sulfated mucins[3,9], which contain longer oligosaccharide chains, as has been shown for tracheal[10] and intestinal[11] mucins. Since mucin oligosaccharide chains are synthesized by adding one monosaccharide residue at a time following the translation of mucin peptide, the sequence of addition of various sugar residues as catalyzed by specific glycosyltransferases determines the final carbohydrate structures. Therefore, increases in carbohydrate-chain length of the mucins obtained from cystic fibrosis patients may be explained[12,13] by changes in the activities of the enzymes that promote carbohydrate-chain growth relative to those which limit or terminate chain elongation. Although several tracheal mucin glycosyltransferases have been characterized in animals[1,6,14–16] and man[8,12], no information is available on mucin α-L-fucosyltransferase, which belongs to the group of glycosyltransferases responsible for limiting or shutting off the carbohydrate-chain growth. This report is part of our effort to characterize the activities of human tracheal epithelial glycosyltransferases involved in the regulation of mucin-oligosaccharide synthesis. We describe herein an α-L-fucosyltransferase (EC 2.4.1.69) that catalyzes reaction (*1*).

$$\text{GDP-L-Fuc} + \beta\text{-D-Gal}p \longrightarrow \text{R}\rightarrow\alpha\text{-L-Fuc}p\text{-}(1\rightarrow2)\text{-}\beta\text{-D-Gal}p\rightarrow\text{R} + \text{GDP} \qquad (1)$$

Kumazaki and Yoshida[17] have recently shown that the properties of an α-(1→2)-L-fucosyltransferase isolated from the milk of secretors are distinctively different from those of the blood group H-specifying-enzyme found in the serum. This finding provides strong biochemical evidence that the secretor gene encoding the α-(1→2)-L-fucosyltransferase in the secretory tissue is a structural rather than a regulatory gene, which is contrary to the model proposed previously[18]. The enzymic properties of human tracheal α-(1→2)-L-fucosyltransferase resemble those reported for human secretor gene-encoded fucosyltransferase found in the milk of secretors[19].

RESULTS AND DISCUSSION

All the enzyme assays were performed in duplicate with and without acceptor under such conditions that the amounts of product formed were a linear function of incubation time and enzyme amount. Except where indicated, linearity was maintained for up to 20% transfer of L-[^{14}C]fucose from GDP-L-[^{14}C]Fuc to the acceptors.

A constant rate of transfer of L-fucose from the donor to the acceptor was maintained for up to 120 min when 3mM ATP was present. Without ATP, the constant reaction rate was maintained for only 60 min, which was extended to

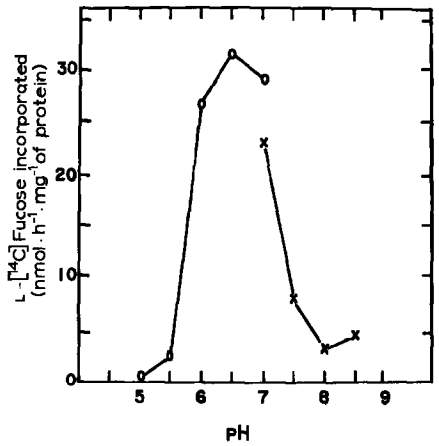


Fig. 1. Effects of pH on human tracheal α-(1→2)-L-fucosyltransferase activity. The reaction mixture containing freezing point-depressing glycoprotein as the acceptor (see the Experimental section for details) was employed except that pH was varied as indicated. Buffers used were morpholinoethanesulfonic acid (—O—O—) and morpholinopropanesulfonic acid (—×—×—). The reaction was terminated after incubation at 37° for 60 min. The fucosylglycoprotein was separated from GDP-L-[^{14}C]Fuc by high-voltage p.e. and quantitatively determined by liquid-scintillation counting.

90 min in the presence of mM ATP. Approximately 80% of GDP-Fuc remained at the end of 120 min when 3mM ATP was present as compared to only 10% left in the absence of ATP. ATP may serve as the competing substrate for nucleotide diphosphatase[20] to minimize the degradation of GDP-Fuc. The optimal pH for the enzyme activity is 6.5 and the activity was greatly decreased at pH 8.0 (Fig. 1). The pH profile is similar to that of human milk α-(1→2)-L-fucosyltransferase, but different from that of human serum α-(1→2)-L-fucosyltransferase. The fucosyltransferase preferred Mn^{2+} or Ca^{2+} to Mg^{2+} or Ba^{2+} as the divalent cation cofactor (Table I). All of these four cations enhanced enzyme activity. However, Fe^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} were strong inhibitors. Triton X-100 at low (0.05–0.1%)

TABLE I

EFFECTS OF DIVALENT CATIONS ON HUMAN TRACHEAL GDP-L-Fuc:β-D-GALACTOSIDE α-(1→2)-L-FUCOSYLTRANSFERASE[a]

Divalent cations	*Relative activity*
None	30[b]
Mn^{2+}, Ca^{2+}	100[c]
Ba^{2+}, Mg^{2+}	74–83
Fe^{2+}	13
Co^{2+}, Zn^{2+}, Cd^{2+}	<1

[a]The concentration of the divalent ion was 25mM and Cl^- was the counter ion. [b]The activity was the same with and without 2mM EDTA. [c]The specific activity of the enzyme using freezing point-depressing glycoprotein as the acceptor was 28 nmol of Fuc transferred per hour per mg of protein. The column method was used to isolate the product. See the Experimental section for detailed experimental conditions.

TABLE II

INHIBITION OF HUMAN TRACHEAL GDP-L-Fuc:β-D-GALACTOSIDE α-(1→2)-L-FUCOSYLTRANSFERASE

Compound (10mM)	*Inhibition (%)*[a]
N-Ethylmaleimide, GMP, GDP, or GTP	98–100
IMP, IDP, ITP, or XDP	60–75
XMP or UTP	30–32
Inosine	10
1-Methylguanosine, 1-methylhypoxanthine, ATP, or dithiothreitol	0

[a]The specific activity of the enzyme in the absence of inhibitor was 29 nmol of L-fucose transferred $h^{-1} \cdot mg^{-1}$ of protein. The freezing point-depressing glycoprotein was used as the acceptor and the column method to isolate the product. See the detailed conditions in the Experimental section.

concentrations increased enzyme activity by 9–14% but inhibited 70–80% of the enzyme activity at concentrations greater than 0.2%. Tween-20 enhanced the enzyme activity by 13–18% at 0.1–1.0% concentrations, but slightly inhibited (9%) the enzyme activity at higher (2%) concentration.

As shown in Table II, *N*-ethylmaleimide, GMP, GDP, and GTP were potent inhibitors, as were IMP, IDP, ITP, XDP, XMP, UTP, and inosine in decreasing order of inhibition. Dithiothreitol did not affect enzyme activity. Like the enzyme specifying the blood group H activity[21], this enzyme activity was completely inhibited by 1–10mM *N*-ethylmaleimide. The effects of guanine, inosine, and xanthine nucleotides on this enzyme activity are similar to those on the intestinal α-(1→2)-L-fucosyltransferase reported by Bella and Kim[22].

The apparent K_m values (in mM) for the glycosyl donor (GDP-L-Fuc) and the

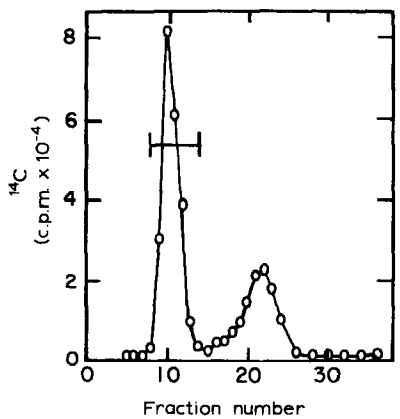


Fig. 2. Isolation of the [^{14}C]fucosylated product on a column (29 × 1.0 cm) of Bio-Gel P-4 (200–400 mesh). The fucosylated product (first peak) prepared in a large batch from 8.8 mg of freezing point-depressing glycoprotein and 2 μmol of GDP-L-[^{14}C]Fuc was separated from the glycosyl donor (second peak). Fraction size was 0.9 mL. The fractions were combined as indicated (I——I).

acceptors, freezing point-depressing glycoprotein and phenyl β-D-galactopyranoside, were 0.29, 5.70, and 25.4, respectively.

A large batch of the product was prepared by use of the freezing point-depressing glycoprotein as acceptor and then isolated by chromatography on Bio-Gel P-4 (see Fig. 2); ~48% of the L-[^{14}C]fucose was transferred to the acceptor. Following the release of the oligosaccharides by alkaline borohydride treatment[23], only one radiolabeled oligosaccharide was detected in Bio-Gel P-2 column chromatography (Fig. 3). The yield of purified ^{14}C-labeled oligosaccharide was 39% of the reaction product, which is similar to the result obtained for the action of the mucin β-(1→6)-*N*-acetyl-D-glucosaminyltransferase on freezing point-depressing glycoprotein[13].

The aforementioned L-[^{14}C]fucosyloligosaccharide cochromatographed with the standard trisaccharide, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol, on Bio-Gel P-2 column chromatography (Fig. 3), in paper chromatography in 6:4:3 (v/v) butanol–pyridine–water, and in g.l.c. When lactose was used as the acceptor, only one peak corresponding to monofucosyllactose was observed on Bio-Gel P-2 column chromatography. When the ^{14}C-labeled trisaccharide isolated from the L-[^{14}C]fucosylated freezing point-depressing glycoprotein was subjected to *Turbo cornutus* α-L-fucosidase treatment, the radiolabel component was completely cleaved off, and it cochromatographed with standard L-fucose on Bio-Gel P-2, suggesting that the fucosyl group was in the α-L anomeric configuration.

G.l.c. analysis, on a 3% OV-225 column at 170°, of the partially methylated alditol acetates prepared from the trisaccharide revealed three major carbohydrate peaks having retention times relative to that of 1,6-di-*O*-acetyl-2,3,4,6-tetra-*O*-

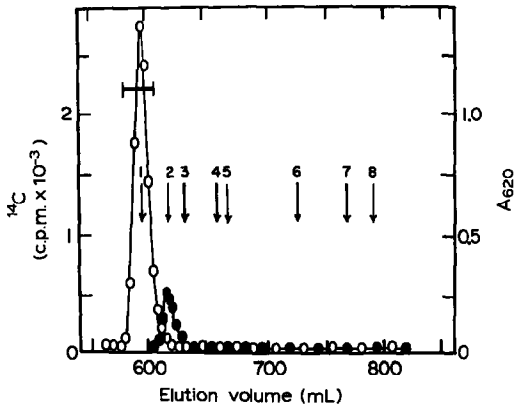
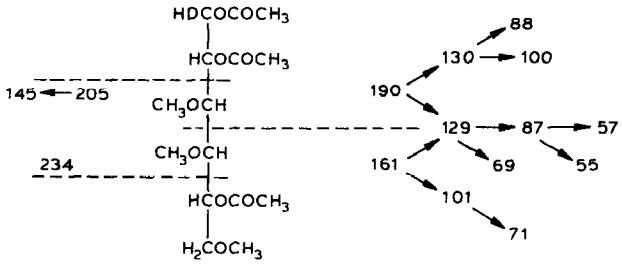


Fig. 3. Chromatography of the oligosaccharides obtained by alkaline borohydride treatment of the [^{14}C]fucosylated freezing point-depressing glycoprotein on a column (190 × 2.5 cm) of Bio-Gel P-2 (—400 mesh). The column was eluted with distilled water at the rate of 27 mL/h. The void volume was 320 mL and the fractions were monitored for radioactivity (—○—○—) and neutral sugar (—●—●—) content. Standards: (1) α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol, (2) β-D-Gal*p*-(1→3)-D-GalNAcol, (3) NeuAc, (4) D-GalNAcol, (5) raffinose, (6) lactose, (7) L-Fuc, and (8) D-Gal. The radioactive fractions were pooled as indicated (I——I) and rechromatographed once.

methylgalactitol (8.64 min) of 0.49 (A), 1.89 (B), and 3.19 (C), respectively. The compound of peak A cochromatographed with 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol, that of peak B with 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol, and that of peak C with 3-*O*-acetyl-2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-galactitol. The ratio of the area of each peak to that of peak C was 0.97:0.84:1.00, suggesting that this oligosaccharide was a trisaccharide. Analysis of these peaks by m.s. showed that peak A compound gave three major, primary ion fragments at *m/z* 118, 131, and 162, and three major, secondary ion fragments at *m/z* 89, 101, and 102, indicating that the peak A compound corresponded to an unsubstituted L-fucosyl group. The compound of peak C was confirmed as 3-*O*-acetyl-2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)galactitol by prominent secondary fragments at *m/z* 246, 142, 130, and 101. The assignment of peak B compound as 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol was based on the identification of the major, primary ion fragment at *m/z* 190 (Fig. 4 and Scheme 1). These results indicate that O-2 of the D-galactosyl residue was substituted with an L-fucosyl group. Therefore, the structure of the α-L-fucosyltransferase product was identified as α-L-fucopyranosyl-β-D-galactopyranosyl, which is the structure



Scheme 1. Primary and secondary ion-fragmentation of peak B compound. The secondary fragments are formed from the primary ions by a single or consecutive loss of CH_3CO_2H (60), $CH_3CO_2{}^2H$ (61), $CH_2{=}C{=}O$ (42), CH_3OH (32), and HCHO (30).

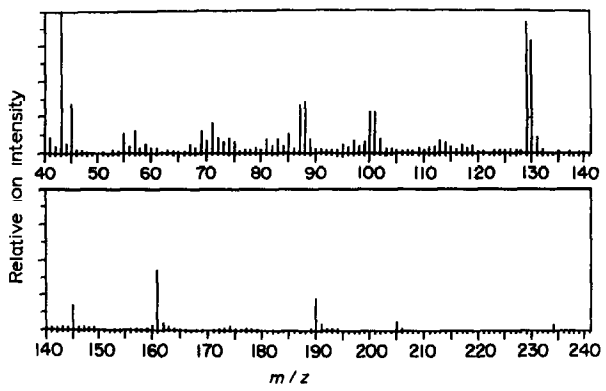


Fig. 4. M.s. fragmentation pattern of peak B compound from g.l.c. column. The primary and secondary ion-fragmentation scheme is illustrated in Scheme 1.

specified by the secretor gene-encoded enzyme found in the secretory tissue as well as by the blood group H enzyme in the serum[4,6,17,22].

EXPERIMENTAL

Materials. — Unless otherwise stated, all the reagents used in this study were reagent grade. The sources of the majority of these reagents were described in previous publications[1,12,13]. GDP-L-[U-^{14}C]Fuc (18.7 mCi/mmol) was purchased from Amersham–Searle Co. (Chicago, IL); *Turbo cornutus* α-L-fucosidase from Miles Scientific (Naperville, IL); and Tri-Sil from Pierce Chemical Co. (Rockford, IL). All human tracheas were obtained within 10 h postmortem from the University Hospitals of Cleveland and the National Diabetes Research Interchange (Philadelphia, PA) and immediately frozen at −20°. Enzyme solutions were prepared by homogenization in 0.25M sucrose solution of the combined epithelial scrapings from these tracheas[12]. The oligosaccharides, β-D-Gal*p*-(1→3)-D-GalNAcol and α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol were isolated from porcine submaxillary mucin according to the procedures of Carlson[23]. Freezing point-depressing glycoproteins 7 and 8 were prepared as described previously[24].

Enzyme assays. — The enzyme activity was measured in an assay mixture (50 μL) containing 50mM morpholinoethanesulfonic acid (pH 6.5), 3mM ATP, 25mM $MnCl_2$, bovine serum albumin (1 mg/mL), 0.25mM GDP-L-[^{14}C]Fuc, and various acceptors including 10mM freezing point-depressing glycoprotein, 160mM lactose, or 70mM phenyl β-D-galactopyranoside. Samples containing no acceptors were used as controls. Enzyme reaction was initiated by the addition of 5–25 μL of enzyme solution containing ~50 μg of protein. Duplicate samples were run for each experimental condition. After incubation at 37° for 60–90 min, the reaction was terminated and the radiolabeled products were separated from glycosyl donors by one of the following three methods.

(*a*) *Column method.* This method was used when macromolecules including freezing point-depressing glycoprotein were used as the acceptors. After termination of the reaction by the addition of ice-cold M NaCl solution (0.4 mL) containing 4mM *N*-ethylmaleimide, an aliquot (0.4 mL) was applied to a Bio-Gel P-4 column. The ^{14}C-labeled product excluded from the column was collected as one fraction with the aid of a multichannel, proportioning pump as described previously[12]. An average value for the duplicate assays of each sample, after subtracting the control value, was expressed as nmol of L-Fuc transferred · h^{-1} · mg^{-1} of protein.

(*b*) *High-voltage paper electrophoresis.* This method was used when lactose or freezing point-depressing glycoprotein was used as the acceptor. After the reaction was stopped by the addition of 20mM *N*-ethylmaleimide (10 μL), the reaction mixture (40 μL) was applied in 5-μL aliquots to a Whatmann No. 3 MM chromatography paper and subjected to electrophoresis in 50mM $Na_2B_4O_7$ buffer (pH 9.1) at 3000 V for 45 min. The radioactive spots corresponding to the

fucosylated product, L-[^{14}C]Fuc, and GDP-L-[^{14}C]Fuc were located with a Packard radiochromatogram scanner. Fucosyllactose moved ~2 cm, fucosylated glycoprotein ~6 cm, L-[^{14}C]Fuc 12 cm, and GDP-L-[^{14}C]Fuc ~19 cm towards the anode. The radiolabeled spots corresponding to the fucosylated products were cut out and the radioactivity measured in toluene scintillator[13] (10 mL). The enzyme activity was calculated as described previously[12].

(*c*) *Paper chromatography.* When phenyl β-D-galactopyranoside was used as the acceptor, the ^{14}C-labeled product was isolated by p.c.[25] in 10:4:3 (v/v) ethyl acetate–pyridine–water for up to 8 h. The ^{14}C-labeled product was located ~20 cm from the origin and quantitatively determined as described earlier.

Preparation of [^{14}C]fucosylated freezing point-depressing glycoprotein and oligosaccharide. — A buffered solution (2 mL; pH 6.5) containing ATP (6 μmol), $MnCl_2$ (40 μmol), morpholinoethanesulfonic acid (100 μmol), bovine serum albumin (2 mg), freezing point-depressing glycoprotein (8.8 mg), GDP-L-[^{14}C]Fuc (2 μmol), enzyme preparation (800 μL), and toluene (1 drop) was incubated for 8 h at 37°. The fucosylated product was separated from the glycosyl donor on a Bio-Gel P-4 column and subjected to alkaline borohydride treatment (50mM NaOH–M $NaBH_4$) for 18 h at 45° according to the procedure of Carlson[23] to release the oligosaccharides. The radiolabeled oligosaccharide was separated from the disaccharide, β-D-Gal*p*-(1→3)-D-GalNAcol, on a column (190 × 2.5 cm) of Bio-Gel P-2 and rechromatographed once.

α-L-Fucosidase treatment of the fucosylated oligosaccharide. — A solution of the fucosylated oligosaccharide (~2 nmol) in 0.1M citrate phosphate buffer (25 μL; pH 5.0) bovine serum albumin (25 μg), and *Turbo cornutus* α-L-fucosidase (0.1 unit) was incubated for 6 h at 37°. L-Fucose (5 mg) and M NaCl (1 mL) were added to the reaction mixture prior to chromatography on a column (190 × 2.5 cm) of Bio-Gel P-2 (—400 mesh).

G.l.c. and g.l.c.–m.s. — To characterize the fucosyloligosaccharide isolated from the fucosylated glycoprotein by g.l.c., the oligosaccharide was mixed with the internal standard, inositol, and then treated with Tri-Sil. After the solvent had been evaporated under a gentle stream of N_2 gas, the trimethylsilylated oligosaccharide was dissolved in CS_2 prior to analysis on a column (3.6 m × 2 mm) of 3% OV-17 (60–80 mesh) with a temperature between 145 and 285° at an increment of 6°/min and then at 285° for 15 min. The peaks were detected by a flame-ionization detector (FID). The retention time for inositol, β-D-Gal*p*-(1→3)-D-GalNAcol, and α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol was 11, 23, and 34 min, respectively.

The permethylated oligosaccharides (~150 nmol) were prepared by the methylation procedure of Stellner *et al.*[26]. After acetolysis in H_2SO_4–acetic acid, reduction with NaB^2H_4, and acetylation with acetic anhydride[13], the partially methylated alditol acetates were separated at 170° on a column (1.8 m × 2 mm) of 3% OV-225. A standard trisaccharide, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol, and lactose were analyzed under the same conditions to facilitate the

identification of each partially methylated alditol acetate by g.l.c. The g.l.c.–m.s. analysis of the three major carbohydrate peaks was carried out by the e.i. technique (70 eV) at the Mass Spectrometry Facility, Department of Biochemistry, Michigan State University, East Lansing, Michigan.

Other methods. — Protein content was estimated by the method of Lowry *et al.*[27] using bovine serum albumin as the standard, and neutral sugars by the anthrone method[28]. Mono- and oligo-saccharides on the p.c. strips were identified by the IO_4–Ag stain[29].

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