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Free oligosaccharides in serum

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ABSTRACT

Glycans are sugars/sugar chains that are usually linked to proteins or lipids. The attachment of glycans often results in alterations of physicochemical/physiological properties of the carrier molecules, e.g., glycosylation of proteins can modulate their fate, intracellular localization, or interaction with cells/other proteins. On the other hand, unconjugated N-glycans (free N-glycans; FNGs) have been identified in the cytosol of eukaryotic cells. The processing pathway of intracellular FNGs has been clarified in recent years, but their biological functions remain unclear. Free oligosaccharides have also been identified in the sera of various animals. Structurally, these extracellular free glycans can be classified into three types: sialyl FNGs, oligomannose-type FNGs, and sialyl lactose/N-acetyllactosamine-type glycans. The extracellular FNGs show different structural features from intracellular FNGs, implying that their mechanism of formation is distinct. This mini-review summarizes current knowledge about the structures and formation mechanisms of free oligosaccharides in serum, and suggests their possible biological functions.

Introduction

Glycans are sugars/sugar chains that universally exist in all living organisms. Among the different type of glycan modifications, the N-linked glycosylation is one of the most common post-translational modifications of proteins that are conserved in eukaryotes, archaea and some bacteria. In mammals, the formation of N-glycoproteins can be divided into three major steps: assembly of N-glycan precursor, i.e., the dolichol pyrophosphate-linked $Glc_3Man_9GlcNAc_2$, at the membrane of endoplasmic reticulum (ER) [1]; transfer of the glycan part from the N-glycan precursor to a nascent peptide via an oligosaccharyltransferase (OST) in the ER [2]; and the processing/remodeling of glycan structures by glycosyltransferases/glycosidases from oligomannose-type N-glycans into hybrid/complex-type N-glycans that often terminated with sialic acid, while the glycoproteins are passing through the secretory pathway to reach their respective destinations [3].

Unconjugated glycans that are structurally related to N-linked glycans, called free N-glycans (FNGs), have been identified in the cytosol, which is segregated from the above-mentioned glycan processing/remodeling events by the lipid bilayer [4–6] (Fig. 1A, B). In mammalian cells, FNGs are believed to be predominantly released from the dolichol-linked N-glycan precursor by hydrolytic activity of OST in the ER [7–11], and are eventually transported into the cytosol [12–15]. FNGs can also be released in the cytosol by a cytosolic peptide: N-glycanase (Ngly1), or alternatively by direct action of a downstream enzyme, i.e., endo-β-N-acetylglucosaminidase (ENGase), from misfolded N-glycoproteins that are destined for proteasomal degradation [16–19]. The cytosolic oligomannose-type FNGs thus formed are further processed by ENGase and a cytosolic α-mannosidase (Man2C1) [20] into a specific $Man_5GlcNAc_1$ structure. These cytosolic processes constitute a "nonlysosomal degradation pathway" for oligomannose-type FNGs [21]. The origin as well as the degradation pathway of oligomannose-type FNGs in the cytosol has been clarified using mouse embryonic fibroblast (MEF) cells derived from wild-type, *Ngly1*-knockout (KO), *Engase*-KO, and *Ngly1/Engase* double-KO mice [8].

In addition to the nonlysosomal degradation pathway for the ER-originated oligomannose-type FNGs, lysosomes are major organelles for catabolism of N-glycoproteins [17]. In normal conditions, glycans are fully digested into monosaccharides in lysosomes. However, a defect in a lysosomal enzyme involved in this process results in the

Abbreviations for glycans: Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; LacNAc, N-acetyllactosamine; Man, mannose; Neu5Ac, N-acetylneuraminic acid; *Other, abbreviations*; ENGase, endo-β-N-acetylglucosaminidase; ER, endoplasmic reticulum; FNGs, free N-glycans; Man2C1, α-mannosidase; Ngly1, peptide:N-glycanase; OST, oligosaccharyltransferase.

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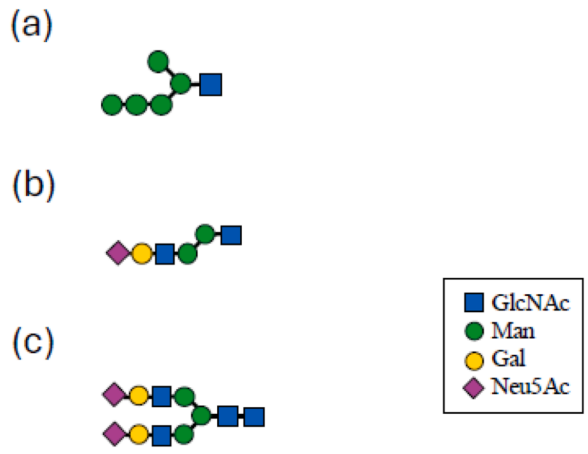

Fig. 1. Representative structures of free N-glycans (FNGs) from the cytosol and serum. (a) The major free cytosolic oligomannose-type N-glycan [8,66]. (b) The major cytosolic sialyl FNG in autophagy-defective cells or cancer cell lines [25,27]. (c) Representative sialyl FNG identified in human serum [32]. The use of monosaccharide symbols follows the Symbol Nomenclature for Glycans system [67]: blue square, N-acetylglucosamine (GlcNAc); green circle, mannose (Man); yellow circle, galactose (Gal); purple diamond, N-acetylneuraminic acid (Neu5Ac).

accumulation of glycan degradation intermediates in lysosomes, causing a large group of genetic disorders collectively called lysosomal storage diseases [17]. In fact, identification of the genetic mutations and the structure of acummulated degradation intermediates in these disorders helped to clarify each enzyme in the lysosomal glycan degradation pathway [22]. Intracellular accumulation of sialyl FNGs with a single GlcNAc at the reducing ends, also called Gn1-type glycans, has been observed in certain cancer cell lines and tissues [23–26] and in autophagy-defective cells [27] (See Fig. 1B for the representative structure). It has been suggested that, considering their structural features, they are likely derived from lysosomal degradation intermediates.

As mentioned above, accumulation of FNGs normally occurs inside cells. However, in recent years, free oligosaccharides have also been identified in the sera of humans and various animals [28–33] (Fig. 1C). Structurally, these serum glycans are quite distinct from the intracellular FNGs (see Fig. 1). We have shown that hepatocytes are likely the source of the free glycans in serum [34]. Herein, we summarize current knowledge about free glycans in serum, i.e., their structural features and catabolic pathways, and we discuss their possible biological functions.

Sialyl free N-glycans in animal sera

Free glycans structurally related to N-linked glycans, i.e., FNGs, in serum were first reported in 2013 [32]. These glycans were shown to carry an N,N′-diacetylchitobiose structure at their reducing termini ("Gn2-type FNGs" for short) (Fig. 2), and they were almost exclusively capped by sialic acid at their nonreducing ends. Because these serum FNGs have similar structural profiles to N-glycans on serum glycoproteins, they were initially hypothesized to be formed from serum glycoproteins during circulation [32]. Later studies showed that the occurrence of serum FNGs is not limited to humans: sialyl FNGs have also been identified in sera from various mammals [28–30,33], chicken [33], and salmon [35]. Interestingly, the major structures of sialyl FNGs differ from species to species. Our improved method for isolation of free oligosaccharides from serum enabled us to show the occurrence of Gn1-type sialyl FNGs, i.e., FNGs with a single GlcNAc at the reducing end [31,33,34] (Fig. 2). Notably, in chicken serum, a significant quantity of partially desialylated FNGs was identified, while such structures are hardly detected in mammalian sera [33]. This could be explained by different specificity of hepatic lectins because mammals carry asialoglycoprotein receptors that recognize desialylated glycans with exposed galactose termini [33,36], while chicken hepatic lectin has a preference toward GlcNAc-terminated glycans [37,38].

There are distinct structural features of bovine serum FNGs from different developmental stages [33]. For example, the disialylated FNG fraction in fetal bovine serum tends to contain tri- or tetra-antennary glycans in addition to the more common biantennary ones, while in adult bovine serum, biantennary glycans are the sole core structure in the equivalent fraction. Moreover, serum glycan patterns also change in certain disease conditions. For example, in patients with liver cancer, a trend of overall increase in the level of the major sialyl FNGs (disialyl biantennary FNGs) was observed [33]. It is thus suggested that sialyl FNGs in sera could reflect the disease and/or developmental status of an animal. In this connection, it is of note that unusual FNGs (i.e., the D-arabinose-containing complex-type) have been identified only in urine samples of patients with cancer [39]. Because urine FNGs are assumed, at least in part, to originate from serum, it would be interesting to examine whether similar noncanonical glycans can be observed in serum of patients with various types of cancer.

Hepatic origin of sialyl FNGs

On the basis of high similarity in the glycan pattern between serum sialyl FNGs and liver-originated glycoproteins [32,33], it was envisaged that hepatocytes are the origin of serum sialyl FNGs. This was later confirmed by our analyses using rat primary hepatocytes [34]. Sialyl FNGs were identified in the culture medium of primary hepatocytes, and they showed similar structural features to those from the serum of the same individual rat. In our study, not only Gn1- and Gn2-type sialyl FNGs but also oligomannose-type FNGs and sialyl lactose/N-acetyllactosamine (LacNAc)-type oligosaccharides were identified both in the culture medium and serum samples (Fig. 2).

Mechanism of release of sialyl FNGs

In mammals, there are two known enzymes that can convert Gn2-type FNGs to Gn1-type ones: the lysosomal chitobiase and the cytosolic ENGase [17]. To understand which enzyme is involved in the formation of Gn1-type FNGs, free oligosaccharides from serum of *Engase*-KO mice have been examined. Surprisingly, the proportion of Gn1-type sialyl FNGs in the total sialyl FNGs was significantly lower in the *Engase*-KO mice than in wild-type mice, but they were not completely absent, implying that both ENGase and lysosomal chitobiase are likely to be involved in their formation [31]. The involvement of lysosomal chitobiase has been further examined by comparing the ratio of Gn1-type FNGs between two groups of animals, those having lysosomal chiotobiase (rat, rabbit, human) and others with no detectable chitobiase activity (cow, goat) [22,40]. While the proportion of Gn1-type FNGs tends to be higher in animals with lysosomal chitobiase activity, there are some exceptions; for example, in our hands, human serum tends to have a very low proportion of Gn1-type glycans [31]. These results collectively indicate that both cytosolic ENGase and lysosomal chitobiase are involved in the formation of serum Gn1-type glycans, and the level of each contribution may vary between species [31].

We could observe the secretion of free oligosaccharides from rat primary hepatocytes, and various inhibitors of glycan processing enzymes were applied to further explore the formation mechanism of serum sialyl FNGs. When castanospermine (CST), an ER α-glucosidase inhibitor, was applied, it was expected that the transport of FNGs from the ER to the cytosol would be impaired [12,13], resulting in the accumulation of FNGs in the ER, followed by their secretion into the culture medium through the secretory pathway [41]. As expected, the addition of CST drastically increased the release of sialyl FNGs into the culture medium of rat primary hepatocytes [34]. Treatment with NGI-1, an inhibitor of OST [42], resulted in decreased release of Gn2-type sialyl

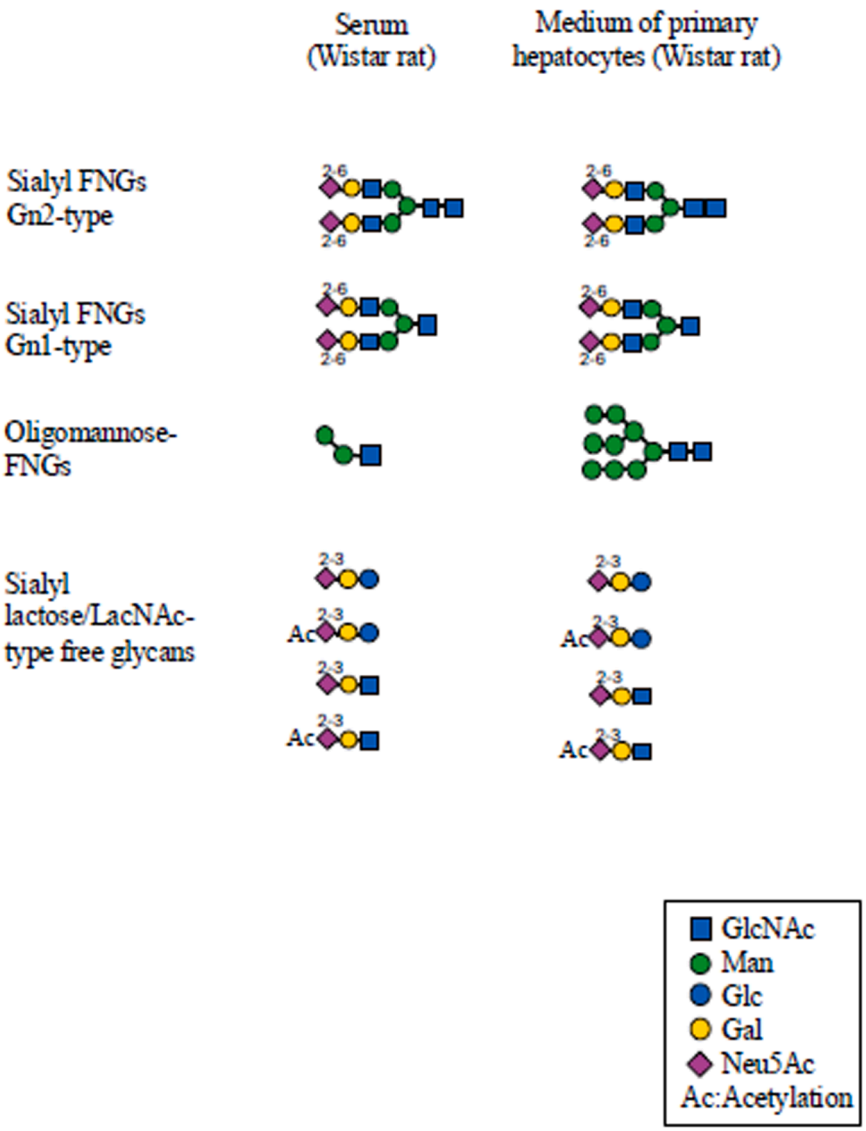

Fig. 2. Representative structures of different types of free glycans from serum and culture medium of rat primary hepatocytes [34]. The Gn1- and Gn2-type sialyl FNGs, oligomannose-type FNGs, and free sialyl lactose/LacNAc-type glycans isolated from a serum sample obtained from Wistar rat (left) [34], and from culture medium of primary hepatocytes prepared from the same Wistar rat (right) [34]. "2–3″ and "2–6″ indicate the linkage of sialic acid (i.e., α2-3-linked or α2-6-linked, respectively). The use of monosaccharide symbols follows the Symbol Nomenclature for Glycans system [67]: blue square, GlcNAc; green circle, Man; blue circle, glucose (Glc); yellow circle, Gal; purple diamond, Neu5Ac; Ac, acetyl group.

FNGs from hepatocytes. These results indicated that OST-derived FNGs could be, at least in part, a source of serum sialyl FNGs. A proposed model is that in primary hepatocytes, OST-derived FNGs are processed into sialyl FNGs by ER/Golgi enzymes while they are passing through the secretory pathway (Fig. 3).

It is noted that this model seems to be specific to primary hepatocytes [34] or HepG2 cells [41] as there is lack of evidence for the above-mentioned events in other cells/tissues. Although the treatment of α-glucosidase inhibitors have also been carried out in previous experiments [12,13,43], only neutral oligomannose-type FNGs was examined in those cases, and therefore whether the similar changes can be observed for sialyl FNGs remains to be examined.

Structures and origin of oligomannose-type FNGs

In addition to sialyl FNGs, oligomannose-type FNGs (Fig. 2) have been detected in various animal sera when using an improved purification protocol [31,34]. The relative level of this type of FNG compared with sialyl FNGs varies among species (0 to 1.3 mol/mol) [31]. The overall structures of serum oligomannose-type FNGs are much smaller than those of cytosolic FNGs [8,27]; $Man_2GlcNAc_1$ (Manα1–6Manβ1–4GlcNAc) and $Man_3GlcNAc_1$ (Manα1-3(Manα1-6)Manβ1–4GlcNAc) are the two major serum oligomannose-type FNGs that have been observed [31]. The relative level of Gn1-type oligomannose-type FNGs (ratio of Gn1- type FNGs to a total of Gn1- and Gn2- type oligomannose-type FNGs) in animals with no detectable chitobiase activity (cow, 0.47; goat, 0.53) tends to be smaller than that in animals having chitobiase activity (rat, 0.95; rabbit, 0.71). Furthermore, a significant increase in the amount of larger ($Man_{6–9}$) mannosidic FNGs has been observed in the serum of *Engase*-KO mice when compared with wild-type mice [31]. This could be explained by the fact that Man2C1, the cytosolic α-mannosidase, prefers Gn1-type glycans over Gn2-type glycans [44], and accordingly, its activity may be significantly compromised in the absence of ENGase. This observation is also consistent with the result of cytosolic FNGs in MEF cells [8]. Taken together, these results suggest that cytosolic FNGs are the major source of oligomannose-type FNGs in the serum. However, how the cytosolic glycans end up in extracellular space remains unknown. Compared with

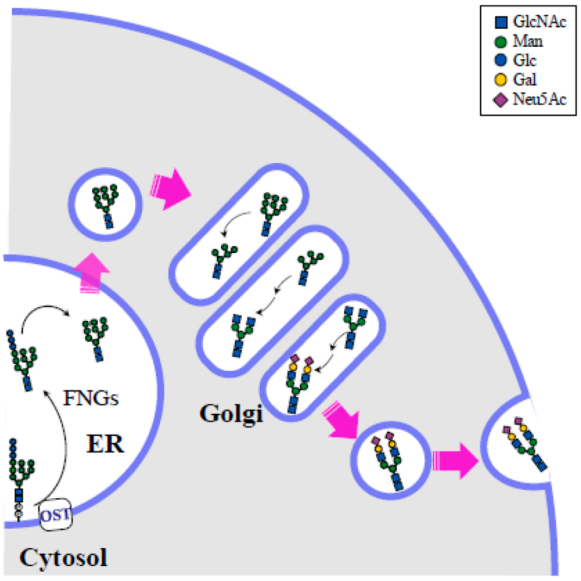

Fig. 3. Proposed model for the release of sialyl FNGs into serum [34]. The FNGs are initially released by the hydrolytic activity of oligosaccharyltransferase (OST) in the endoplasmic reticulum (ER). It is suggested that in primary hepatocytes, the released glycans are transported into the Golgi through vesicular transport, and are then processed from oligomannose-type FNGs into sialyl FNGs by ER/Golgi enzymes before their release outside the cell [34]. The use of monosaccharide symbols follows the Symbol Nomenclature for Glycans system [67]: blue square, GlcNAc; green circle, Man; blue circle, Glc; yellow circle, Gal; purple diamond, Neu5Ac. Figure modified from reference [34].

serum oligomannose-type FNGs, FNGs secreted from primary hepatocytes tend to be larger with more mannose residues, and their secretion is inhibited by treatment with NGI-1 [34]; these results clearly indicate the involvement of OST in their formation.

As regards the difference in the size of oligomannose-type FNGs between serum and the culture medium of primary hepatocytes, it appears that serum α-mannosidase activity can trim the mannoses during blood circulation. The occurrence of serum glycosidase activities was hypothesized previously, and lysosomal enzymes were shown to be their source [45]. We also detected such enzyme activity in rat serum [34]. It was therefore speculated that the oligomannose-type FNGs released by hepatocytes were further processed by α-mannosidases in blood [34].

Sialyl lactose/LacNAc-type free glycans

In addition to the FNGs, small sialyl glycans that were characterized to be sialyl lactose/LacNAc-type free glycans have also been identified in the sera of human, baboon [46], rat, rabbit, goat, bovine [31] and salmon [35]. Our recent experiments have indicated that these glycans are also secreted from hepatocytes [31,34].

Notably, the linkages of sialic acids in FNGs and sialyl lactose/LacNAc-type free glycans from the same serum sample are quite distinct (Fig. 2). For example, most rat serum FNGs contain α2–6 linked N-acetylneuraminic acid (Neu5Ac), whereas most sialyl lactose/LacNAc-type glycans contain α2–3 linked Neu5Ac. These results may be due to the substrate specificity of the sialyltransferases involved the biosynthesis of these glycans. Most identified free sialyl lactose/LacNAc-type glycans are small (i.e., trisaccharides), but larger forms, such as disialyllacto-N-neohexaose, have also been characterized [34].

Similar structures of lactose/LacNAc-type sialyl glycans were also identified in milk oligosaccharides [47–51]. It has been shown that milk oligosaccharides are generated exclusively by the mammary gland because α-lactalbumin, the regulatory subunit of β-galactosyltransferases for the formation of lactose, has been reported to occur only in this tissue [52,53]. Our results suggest the presence of an unknown biosynthesis mechanism for the formation of sialyl lactose/LacNAc-type glycans in hepatocytes [34].

Occurrence of other lactose-based neutral free glycans

Aside from the sialyl lactose/LacNAc glycans, lactose-based free glycans with ABO blood group-specific antigens have also been identified through a comprehensive glycomic analysis of human serum and cerebrospinal fluid [30]. Whether those free glycans are formed by the similar pathway as the hepatocytes-derived sialyl lactose/LAcNAc-type glycans, however, remains to be clarified.

O-acetylation of sialic acid on serum free glycans

In sera from wistar rat [34] and salmon [35], partial 9-*O*-acetylation of sialic acid has been identified both on sialyl lactose/LacNAc-type glycans and sialyl FNGs. This modification may be specific for certain species as such modification was not detected in other serum samples [31,33]. The *O*-acetylation on sialic acid has been widely observed, and it has been reported that the level of *O*-acetylated sialic acid is correlated with development, cancer, immune regulation, and microbiome [54]. It is thus curious to know whether this modification on serum free glycans occurs as needed under certain conditions.

Possible biological functions of free glycans in serum

In sharp contrast to protein-bound glycans, for which disease-specific glycoforms have been identified [55–58], the biological functions of free glycans remains largely unknown, except for some suggested roles for milk oligosaccharides [59,60]. The amount of serum sialyl free N-glycans varies from species to species, and reported results varies from 0.07 to 3.3 nmol/mL in sera of different species [31,33,35], and for the amount of sialyl lactose/LacNAc-type free glycans, it was about 1.4–6 nmol/mL [31,35,46]. The level of free glycans therefore can be considered adequate to exert a biological effect in the blood; for example, concentration of serum soluble Siglec-1 has been reported to range from 1.28 to 276.1 ng/mL [61], which is around 6–1400 fmol/mL. With respect to the possible roles of free glycans in serum, we hypothesize that they may function through interactions with host lectins or invading bacteria/viruses [62]. For instance, a possible function of those glycans is to modulate the activity of host lectins to fine-tune and avoid excess inflammatory responses. Another possibility arises because, during bacterial/viral invasion, glycan–lectin interactions play a very important part in the interaction of the pathogen with host cells [63–65]; the free glycans in serum may therefore function to inhibit those interactions by serving as a “decoy” to bind to viral/bacterial lectins before they recognize cell-surface glycans. Further studies are required to clarify the precise roles of free oligosaccharides in serum.

Perspectives

While the occurrence of free glycans in serum has clearly been demonstrated, questions regarding their mechanisms of biosynthesis still remain to be answered. For example, how cytosolic ENGase- or lysosomal chitobiase-processed Gn1-type FNGs end up in serum remains to be unveiled. Moreover, how precisely sialyl lactose/LacNAc-type glycans are synthesized in the liver needs to be clarified. Furthermore, while a considerable portion of sialylated free glycans are *O*-acetylated [34,35] it is unknown whether this modulates their biological functions. In any event, because free oligosaccharides in serum have been shown to be secreted from hepatocytes, we can assume that their amount and structures reflect the functionality of the liver. Therefore, it will be important to analyze the free glycans in serum under various disease conditions because they may provide excellent hepatic disease-specific biomarkers.

CRediT authorship contribution statement

Chengcheng Huang: Writing – review & editing, Writing – original draft, Conceptualization. **Akinobu Honda:** Writing – review & editing. **Tadashi Suzuki:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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