The Centrally Acting β1,6N-Acetylglucosaminyltransferase (GlcNAc to Gal)

FUNCTIONAL EXPRESSION, PURIFICATION, AND ACCEPTOR SPECIFICITY OF A HUMAN ENZYME INVOLVED IN MIDCHAIN BRANCING OF LINEAR POLY-N-ACTEYLLACTOSAMINES

(Received for publication, May 8, 1998, and in revised form, July 22, 1998)

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In the present experiment the cDNA coding for a truncated form of the β1,6N-acetylglucosaminyltransferase responsible for the conversion of linear to branched polylactosamines in human PA1 cells was expressed in SF9 insect cells. The catalytic ectodomain of the enzyme was fused to glutathione S-transferase, allowing effective one-step purification of the glycosylated 67–74-kDa fusion protein. Typically a yield of 750 μg of the purified protein/liter of suspension culture was obtained. The purified recombinant protein catalyzed the transfer of GlcNAc from UDP-GlcNAc to the linear tetrasaccharide Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc, converting the acceptor to the branched pentasaccharide Galβ1→4GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4GlcNAc as shown by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, degradative experiments, and 1H NMR spectroscopy of the product. By contrast, the recombinant enzyme did not catalyze any reaction when incubated with UDP-GlcNAc and the trisaccharide GlcNAcβ1→3Galβ1→4GlcNAc. Accordingly, we call the recombinant β1,6-GlcNAc transferase cIGnT6 to emphasize its action at central rather than peridistal galactose residues of linear polylactosamines in the biosynthesis of blood group I antigens. Taken together this in vitro expression of I-branching enzyme, in combination with the previously cloned enzymes, β1,4galactosyltransferase and β1,3N-acetylglucosaminyltransferase, should allow the general synthesis of polylactosamines based totally on the use of recombinant enzymes.

Human embryonal carcinoma cells of line PA1 express large amounts of polylactosamines covalently bound to proteins (1–4). Human and murine embryonal carcinoma cells synthesize polylactosamines, which resembled each other in size, mono- saccharide composition (5), and branching (6, 7). In human erythrocytes, linear poly-N-acetylactosamines (Galβ1→4GlcNAcβ1→3)n, are converted to branched arrays, Galβ1→4GlcNAcβ1→3(Galβ1→4GlcNAcβ1→6)Galβ1→R after birth (8, 9). These two types of poly-N-acetyllactosamine backbones represent I and I blood group antigens, respectively (8, 9).

Two protein enzymes, IGN16, appear to be involved in the biosynthesis of branched polylactosamine backbones. One of them, dIGN16, adds β1,6N-acetylglactosamyl to the peridistal galactose residue of GlcNAcβ1→3Galβ1→4GlcNAcβ1→OR, forming GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4GlcNAcβ1→OR (10–14). This enzyme does not act at the central galactose of GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc (15). The other IGN16 (cIGN16) catalyzes the generation of β1,6N-acetylgalactosamyl branches at the central galactose residue of Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→OR (14, 16, 17) and at both central galactose residues of Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc (18). This enzyme does not react at the GlcNAcβ1→3Galβ1→4GlcNAcβ1→OR (15).

The cDNA coding for the enzyme responsible for the key reaction in the biosynthesis of the branched polylactosamine backbone has been isolated (19), but it is not known whether it codes for a cIGN16 or a dIGN16 enzyme or perhaps for an unknown branch-generating enzyme. In the present experiments, a fusion protein representing the catalytic ectodomain of the branch-forming enzyme and glutathione S-transferase was functionally expressed in Baculovirus-infected insect cells and purified. Analysis of the substrate specificity of the purified recombinant enzyme showed that it possesses the activity of the cIGN16 type, but not of the dIGN16 type. The data suggest that the recombinant cIGN16 is able to transfer multiple GlcNAc branches to long linear polylactosamines, a prerequisite for improving enzyme-assisted in vitro synthesis of a type of multivalent sialyl Lewis x glycans (21, 22) that are high affinity inhibitors of lymphocyte L-selectin.

EXPERIMENTAL PROCEDURES

Materials—The human PA1 cell β1,6N-acetylglucosaminyltransferase that generates branches to polylactosamine backbones (IGN16) has been cloned previously and sequenced (19). The following materials were purchased from indicated sources: Pfu polymerase (Stratagene), oligonucleotides A and B (Amersham Pharmacia Biotech), T4 ligase (Promega), Escherichia coli strains JM 105 and XL-1Blue (ATCC), BamHI and EcoRI (Promega), Baculovirus transfer vector pAcSec20T, linearized BaculoGold DNA, pAGHLT XyIE control plasmid, and transfection buffers A and B (PharMingen), SF9 cells (Invitrogen), SF-900 medium, gentamycin, penicillin, streptomycin (all from Life Technologies, Inc.), insect cell lysis buffer, protease inhibitor cocktail, glu-
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tanno-agarose beads, phosphate-buffered saline wash buffer, GST elution buffer, glutathione powder (PharMingen), Microcon 30 concentrators (Amicon), plastic ware (Greiner), monoclonal mouse anti-GST (Zymed Laboratories Inc.), ECL reagents (Amersham Pharmacia Biotech).

General Procedures—Sf9 cells were grown at 27 °C in SF-900 medium supplemented with 10 μg/ml gentamycin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum in either 10-cm Petri dishes, six-well plates, or 75-cm² tissue culture flasks. Restriction endonuclease reactions, DNA ligations, bacterial transformations plasmid isolations, RNA isolations, Northern blots, Western blots, ECL reactions, and Coomassie staining were performed by standard methods.

Construction of the Baculovirus Transfer Vector pAcSecG2T-IGN6—The truncated segment coding for residues 26–400 of human IGN6 was synthesized with polymerase chain reaction (PCR) using Ffu polymerase. The IGN6 cDNA (19) in pCDNAI vector was used as a template, and oligonucleotides A (5′-CAAGAAGATCCATTTTGG-GGAGATCCAGGC) and B (5′-GGATGAATCTCATATATACGCGTGGGTGTATGCG) as primers. Oligonucleotide A created a BamHI site to the 5′ end of the truncated IGN6 DNA, and oligonucleotide B created an in-frame stop codon and an EcoRI site to its 3′ end. For expression in insect cells as GST fusion protein, the amplified IGN6 PCR product was subcloned into the plasmid pAcSecG2T (PharMingen) downstream of the BarnHI and XhoI sites. The construct lacks the section of DNA encoding the cytoplasmic N terminus and transmembrane region of IGN6.

Expression of GST-IGN6 in the Baculovirus/Insect Cell System—Transfer vector pAcSecG2T-IGN6 (4.4 μg) and BaculoGold Baculovirus-linearized DNA (0.5 μg) were co-transfected into confluent Sf9 cells and incubated for 3 days at 27 °C. GST-IGN6 virus progeny was isolated using plaque assay and amplified three times. The recombinant virus was stored as a stock solution (4 × 10⁶ plaque-forming units/ml) at 4 °C in SF-900 medium containing supplements and 10% fetal calf serum. For activity assays the recombinant enzyme was stored a few days at −20 °C without loss of activity.

Purification of GST-IGN6—Microscale purification was performed: 2.0 × 10⁷ Sf9 insect cells were infected or not infected with recombinant Baculovirus (4 plaque-forming units/ml) and incubated at 27 °C for 3 days. The cells were lysed on ice for 45 min with the lysis buffer containing protease inhibitors and preclarified by centrifuging at 40,000 × g for 30 min to pellet the cellular debris. Preclarified lysates were loaded into the glutathione bead column after which the column was washed several times with phosphate-buffered saline wash buffer. The fusion protein was eluted with the GST elution buffer containing glutathione (5 mM). Glutathione was removed by dialyzing against 50 mM Tris-HCl (pH 8.0) or by washing the eluates several times in Microcon 30 concentrators.

Oligosaccharides—The acceptor oligosaccharides (Table I) were synthesized as described: the trisaccharide GlcNAcβ1–3Galβ1–4GlcNAc (23), the tetrasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (24), the pentasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4Fucα1–3GlcNAc (25), and the hexasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4Fucα1–3GlcNAcβ1–3Galβ1–4GlcNAc (22). The marker Galβ1–4GlcNAcβ1–3GlcNAc1–6Galβ1–4GlcNAc was synthesized as described in Ref. 17.

Glycosyltransferase Reactions—The IGN6 reactions with the purified recombinant enzyme were performed by incubating the acceptor oligosaccharides (1–40 nmol) and UDP-GlcNAc (1.4 μmol) with 1 μg of the recombinant enzyme for 120 h in a total volume of 10 μl of a solution containing 200 mM MOPS (pH 7.0), 20 mM EDTA, 0.5 mM ATP, 0.28 mM dithiothreitol, 8 mM NaN₃, 10% glycerol, 0.2% bovine serum albumin, the acceptor oligosaccharide and the cell lysate (450 μg total protein) in a total volume of 50 μl. The cell lysate was prepared by incubating equal volumes of the cells and the lysis solution (1.8% NaCl, 1% Triton X-100, and the protease inhibitors).

Chemotrophic Methods—Paper chromatographic runs of desalted radiolabeled saccharides were performed on Whatman III H Chr paper with the upper phase of 1-butanol/acetatic acid/water (4:1.5 v/v). Radioactivity on the chromatograms was monitored using Oprisincit (Wallac, Turku, Finland) as scintillant. Marker lanes of malto-oligosaccharides, lactose, and galactose on both sides of the sample lanes were stained with silver nitrate.

Degradative Experiments—Digestions with endo-β-galactosidase from Bacteroides fragilis (EC 3.2.1.103; Boehringer Mannheim, Mannheim, Germany) were performed according to Ref. 16, parallel control reactions cleaved over 90% of radiolabeled GlcNAcβ1–3Galβ1–4GlcNAc. Digestions with jack bean (exo-β)-galactosidase were performed as described in Ref. 26.

1H NMR Experiments—The 1H NMR experiments were carried out as described (17).

RESULTS

Construction of pAcSecG2T-IGN6—IGN6 is the β1,6-GlcNAc Transferase That Generates Branches to Poly-N-acetyllactosamine backbones in human PAIL cells (19). The truncated IGN6 (amino acids 26–400, Fig. 1A), encoding for the stem and the Golgi lumenal regions of native IGN6, was synthesized by PCR. It was inserted downstream of the very late polyhedrin promoter, gp67 signal sequence, and GST coding region of the vector pAcSecG2T, between the BarnHI and EcoRI restriction sites in the cloning site to form the transfer vector pAcSecG2T-IGN6 (Fig. 1B).

Expression of Human GST-IGN6 in Sf9 Insect Cells—Sf9 insect cells were co-transfected with the pAcSecG2T-IGN6 transfer vector together with the linearized BaculoGold Baculovirus DNA. Northern blot analysis from the infected cells indicated that a new RNA transcript of the size of 2.3 kilobases hybridizing with the full-length IGN6 cDNA was present (Fig. 2A). This de novo expressed transcript was first detected at 48 h, an increase in the level was measured until 72 h postinfection.

Expression of the recombinant fusion protein GST-IGN6 was monitored by Western blot analysis with a monocular anti-GST antibody. Proteins in the cell culture media and lysates from both uninfected as well as from infected cells were separated. While no immunoreactive bands were present in the samples from the culture media, two broad bands at 67 and 74
kDa were detected by anti-GST antibody in samples prepared from cell lysates at 48–96 h after infection (Fig. 2B).

The fusion protein GST-IGnT6 has five potential N-glycosylation sites. To study them we infected Sf9 cells with recombinant virus following by treatment with tunicamycin, an inhibitor of N-glycosylation. After tunicamycin treatment only two bands centered at 67 kDa were detected with the anti-GST antibody in the Western blot (Fig. 3). These data showed that the IGnT6 was N-glycosylated in the Sf9 cells, and the size heterogenicity was at least partially due to differences in N-glycosylation.

Purification of Recombinant GST-IGnT6—A one-step purification of the recombinant GST-IGnT6 was achieved by affinity chromatography using glutathione-agarose beads. Samples of the cell lysate and the purified protein were run in SDS-PAGE and stained with Coomassie Blue. A major band was observed at 67 kDa in the lane of the purified protein; minor bands were visible at 58 and 76 kDa (Fig. 4). The yield of the purified fusion protein was typically 750 μg/10⁹ infected Sf9 cells present in 1 liter of the suspension culture.

Characterization of the GlcNAc Transferase Activity of the Recombinant GST-IGnT6—The polylactosamine acceptors used in these experiments are collected in Table I. The functionality of the recombinant GST-IGnT6 was studied first by using Sf9 cell lysates. In a typical experiment, a lysate was incubated with radiolabeled trisaccharide GlcNAcβ1–3[^14C]Galβ1–4GlcNAc and UDP-GlcNAc. Neither a tetrasaccharide-like product nor any other product besides the starting trisaccharide was detected by paper chromatography of the neutral oligosaccharides of the incubation mixture (Fig. 5A). By contrast, similar experiments repeatedly converted significant amounts of the radiolabeled tetrasaccharide [³H]Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc into a product that migrated like a pentasaccharide, suggesting the presence of cIGnT6 activity (data not shown, see below).

The functionality of the recombinant enzyme GST-IGnT6 was confirmed by incubating it with UDP-GlcNAc and [³H]Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc. This reaction mixture contained a radiolabeled oligosaccharide product, which co-migrated with the authentic Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (Fig. 5B). A partial reaction had taken place, yielding 28% of a pentasaccharide (peak 1) and leaving 72% of the tetrasaccharide acceptor (peak 2) intact.

FIG. 2. Expression of GST-IGnT6 in Sf9 insect cells. A, total RNA was extracted from Sf9 cells either noninfected or infected with the recombinant Baculovirus for 24–72 h, blotted on the membrane, and probed with full-length IGnT6 cDNA. B, proteins in the culture media and cell lysates from noninfected and infected cells were separated in SDS-PAGE and transferred to membrane. Two broad bands of recombinant protein around 67 and 74 kDa were detected by ECL using anti-GST antibody.

FIG. 3. Effect of tunicamycin on the expression of GST-IGnT6. Sf9 cells noninfected or infected with recombinant Baculovirus for 24 h were either treated or nontreated with tunicamycin and further incubated at 27 °C for 48 h. The recombinant protein was detected by ECL using anti-GST antibody.

FIG. 4. Purification of GST-IGnT6. Sf9 cells infected with the recombinant virus were lysed, precleared, and applied to a column filled with glutathione beads. Recombinant protein was eluted with free glutathione and dialyzed. Samples of a cell lysate as well as purified recombinant protein, both containing 15 mg of protein, were run in SDS-PAGE and stained with Coomassie Blue.
This was confirmed by MALDI-TOF mass spectrometry, performed with sample from another similar but more exhaustive reaction mixture; the spectrum revealed the presence of 49% of a pentasaccharide Galβ1GlcNAc3 in addition to 51% of the acceptor tetrasaccharide (Fig. 6A).

To characterize the pentasaccharide product, the radiolabeled glycan was first incubated with jack bean (exo)-β-galactosidase, which released all tritium label in the form of free [3H]Gal (Fig. 7A). This implies that the new GlcNAc of the pentasaccharide was not transferred to the distal, tritium-containing galactose residue of the tetrasaccharide acceptor, as this would not have been susceptible to (exo)-β-galactosidase. Hence, the reaction had been different from the β1,6-GlcNAc transfer to the terminal galactose described in other laboratories (12, 27–30). Another enzymatic digestion was performed with endo-β-galactosidase, which cleaves the internal β-galactosidic linkage of the tetrasaccharide acceptor (27) and other linear polylactosamines, but does not hydrolyze the branched Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (B).

This was confirmed by MALDI-TOF mass spectrometry, performed with sample from another similar but more exhaustive reaction mixture; the spectrum revealed the presence of 49% of a pentasaccharide Galβ1GlcNAc3 in addition to 51% of the acceptor tetrasaccharide (Fig. 6A).
polylactosamine branching in PA1 cells converts 3-substituted galactoses to 3,6-disubstituted units. These combined data imply that the pentasaccharide product generated by the recombinant enzyme in the present experiments was almost certainly Galβ1–4GlcNAcβ1–3(GlcNAc1–6)Galβ1–4GlcNAcN.

The purified recombinant GST-IGnT6 catalyzed the GlcNAc transfer also to the linear Galβ1–4GlcNAcβ1–3’Galβ1–4GlcNAcβ1–3’Galβ1–4GlcNAc, generating in a partial reaction nonasaccharides of the type (GlcNAc)1–β1–4GlcNAcβ1–3Galβ1–4GlcNAc and decasaccharides of type (GlcNAc)2–β1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3GlcNAcβ1–3Galβ1–4GlcNAc as shown by MALDI-TOF mass spectrometry (Fig. 6B). The nona- and decasaccharide products represented mixtures of isomers carrying one and two GlcNAc branches on the linear octasaccharide acceptor. Hence, it appears likely that the recombinant cIGnT6 shares with the enzymes of mammalian serum (18) and hog intestine (46) the capacity to form multiple branches on long linear polylactosamine acceptors.

The recombinant GST-IGnT6 was unable to transfer GlcNAc to the pentasaccharide [3H]Galβ1–4GlcNAcβ1–3Galβ1–4(Fucα1–3)GlcNAc, as assessed by paper chromatography, while the control reaction performed with the fucose-free tetrasaccharide [3H]Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc gave a 16% yield of the radiolabeled pentasaccharide product (data not shown). Hence, the recombinant enzyme shares with the rat serum cIGnT6-enzyme (18) in the inability to act at the inner galactose of the fucosylated pentasaccharide.

Taken together, all properties of the recombinant GST-IGnT6 that were tested in the present experiments were qualitatively similar to those of the cIGnT6 activity of rat serum.

**DISCUSSION**

The present experiments describe successful functional expression of a truncated form of a human β1,6-GlcNAc transferase as a fusion protein with glutathione-S-transferase in Baculovirus-infected insect cells and provide evidence that the purified recombinant enzyme represents a cIGnT6 that catalyzes transfer to centrally located galactose residues of linear polylactosamine chains. The cDNA expressed was originally isolated from human embryonal carcinoma cells of line PA1, where it was shown to code for the enzyme responsible for the biosynthesis of branched polylactosamine backbones (19).

**TABLE II**

| Residue | Authentic pentasaccharide synthesized by cIGnT6 of rat serum (32) | Pentasaccharide synthesized by cIGnT6 of PA1 cell lysates (20) | Pentasaccharide synthesized by recombinant cIGnT6 in a Sf9 cell lysate |
|---------|------------------------------------------------------------------|----------------------------------------------------------------|------------------------------------------------------------------|
| H-1 GlcNAc-1 (α) | 5.212 | 5.212 | 5.213 |
| Gal-2 | 4.454 | 4.453 | 4.453 |
| GlcNAc-3 | 4.701/4.696 | 4.701/4.696 | 4.701/4.696 |
| Gal-4 | 4.481 | 4.480 | 4.480 |
| GlcNAc-5 | 4.585 | 4.585 | 4.585 |
| Gal-2 | 4.149 | 4.148 | 4.147 |
| GlcNAc-1 (β) | 2.056 | 2.055 | 2.055 |
| GlcNAc-3 | 2.032 | 2.032 | 2.032 |
| GlcNAc-5 | 2.051 | 2.050 | 2.051 |

For monosaccharide denotation, see Fig. 8.

The two values given correspond to the two anomers of the pentasaccharide.

- $^c_{3J_{2,3}}$ 8.5 Hz
- $^d_{3J_{2,3}}$ 8.5 Hz
- $^e_{3J_{2,3}}$ 8.5 Hz
The present data show that the CDNA does not code for an enzyme that transfers at the distally located galactose units at the nonreducing termini of the acceptor chains. The data show also that the CDNA does not code for dIGnT6, a branching enzyme that acts at peridistal galactoses of polylactosamine chains of the type GlcNAcβ1–3Galβ1–4GlcNAcβ1–OR. Instead, the data imply that the CDNA codes for a branching enzyme that transfers to midchain galactoses of polylactosamines; the presence of at least one complete N-acetyllactosaminyl unit, bonded to position 3, appears to be necessary for the galactose residues reacting with the purified recombinant cIGnT6.

Our recent observations show that PA1 cell lysates contain cIGnT6 rather than dIGnT6 activity (20). Hence, the elimination of the cytoplasmic and the membrane binding segment from the recombinant cIGnT6 of the present experiments was probably not associated with major changes in the substrate specificity. Consequently, it is worth noting that the recombinant cIGnT6 shares several features with the soluble cIGnT6 enzymes present in mammalian serum (18, 31) and with the specificity. Consequently, it is worth noting that the recombinant cIGnT6 enzyme of the present experiments was probably not associated with major changes in the substrate specificity.

The present study demonstrates that cIGnT6 adds β,6-linked N-acetylgalactosamine to a linear poly-N-acetyllactosamine as shown in Fig. 9A. In this biosynthetic pathway, the addition of I branch does not occur at the termini of elongating polyn-acetyllactosamine. It is expected that the addition of I branches proceeds randomly along preformed poly-N-acetyllactosamine chains in human and rabbit erythrocytes (33, 34). Analysis of PA1 cells (7) demonstrated that their poly-N-acetyllactosaminyl backbone have uniformly short branches, consisting of single N-acetyllactosaminyl units with or without terminal substituents. Such arrays could result from a relatively late action of cIGnT6 on preformed i-type chains (Fig. 9A). In contrast, dIGnT6 is expected to form branched poly-N-acetyllactosaminyl backbones in association with the chain growth, leading occasionally to the formation of branched branch arrays of N-acetyllactosaminyl units in the multiply branched polylactosamines (Fig. 9B). Such structures may be synthesized in gastrointestinal cells and Novikoff cells where dIGnT6 activity has been detected (10, 35). Indeed, hog gastric mucosa contains an octadecaneramic tetra-α-npecto-lipid-bound polylactosamine (45) that resembles strikingly the branched branches array of seven N-acetyllactosaminyl residues that we have synthesized in vitro by using dIGnT6 in combination with GnT3 and GalT4 (39, 41, 42).

As shown previously, human granulocytes contain heavily fucosylated poly-N-acetyllactosamines such as R1–Galβ1–4(Fucα1–3)GlcNAcβ1–R2, and these side chains do not contain any I branching (36, 37). Our present study suggests that the lack of I branching in granulocytes poly-N-acetyllactosamines could be due to the inhibition of cIGnT6 by α,1,3-fucosyl residues. Alternatively, human granulocytes may not express IGN6. On the other hand, the termini of I structures such as Fucα1–2Galβ1–4GlcNAc(Fucα1–2Galβ1–4GlcNAcβ1–6)Gal (33, 34) provide the H antigen or its modifications, the A and B antigens, on two neighboring N-acetyllactosamines. Such bivalent antigenic structures function as much better ligands for anti-ABO antibodies than single antigenic structures (38). In the same vein, it has been demonstrated that multivalent sialyl Lewis polylactosamines at very low concentrations can inhibit L-selectin-mediated lymphocyte binding to the endothelium of lymph nodes (39) and rejecting organ transplants (21, 40–42).

In combination with the previously cloned enzymes GalT4 (43, 44) and GnT3 (45), the purified recombinant cIGnT6 should allow general polylactosamine synthesis that is totally based on the use of recombinant enzymes. Further studies on synthesis of biontive poly-N-acetyllactosamines with long, branched backbones are of great interest because such oligosaccharides are expected to be powerful carbohydrate-based antagonists of selectins and other sugar-binding proteins.

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