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Biochemical and Genomic Analysis of Neoculin Compared to Monocot Mannose-Binding Lectins

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Neoculin occurring in an edible tropical fruit is a heterodimeric protein which has both sweetness and a taste-modifying activity that converts sourness to sweetness. Both the primary and the overall tertiary structures of neoculin resemble those of monocot mannose-binding lectins. This study investigated differences in biochemical properties between neoculin and the lectins. Structural comparison between the mannose-binding sites of lectins and the corresponding regions of neoculin showed that there is at least one amino acid substitution at each site in neoculin, suggesting a reason for the lack of its mannose-binding ability. This was consistent with hemagglutination assay data demonstrating that neoculin had no detectable agglutinin activity. DNA microarray analysis indicated that neoculin had no significant influence on gene expression in Caco-2 cell, whereas kidney bean lectin (*Phaseolus vulgaris* agglutinin) greatly influenced various gene expressions. These data strongly suggest that neoculin has no lectin-like properties, encouraging its practical use in the food industry.

KEYWORDS: Neoculin; sweetener; lectin; DNA microarray

INTRODUCTION

In recent years there has been an increasing demand for low-calorie and nonglycemic sweeteners. Synthetic sweeteners including aspartame, saccharin, acesulfame-K, sucralose, cyclamate, neohesperidine DC, and thaumatin, a plant-derived protein, are widely used by those suffering from diseases associated with excessive carbohydrate consumption, as well as health-conscious people trying to control their carbohydrate intake with the aid of artificial low-calorie sweeteners (*1–3*). On the other hand, there has been an increase in demand for more "natural" food products. Several studies have warned that some artificial sweeteners have unfavorable side effects, for example, carcinogenicity, and their safety is still controversial (*2, 4–6*).

Six proteins—brazzein, thaumatin, monellin, neoculin (formally known as curculin), mabinlin, and pentadin—are known to elicit a sweet taste response in humans. Sweet proteins generally have strong sweetness ranging from 100 to 3000 times greater than that of sucrose on a weight per weight basis and are thus very good candidates as low-calorie, nonglycemic sweeteners (*2*). Among these molecules, neoculin, which occurs in the fruit of *Curculigo latifolia* that grows in Malaysia, is the only known protein that elicits a sweet response and has taste-modifying activity to convert sourness to sweetness (*7, 8*). Sour substances are tasted as sweet in the presence of neoculin. This type of taste-modifying activity is also evoked by miraculin, which, however, has no sweetness by itself (*9*).

Our group has been investigating the mechanism of the sweetness of neoculin and its taste-modifying activity and has succeeded in defining the interaction with human sweet taste receptor hT1R2-hT1R3 expressed in HEK293T cells together with Gα protein (*10, 11*). The important role of His residues in taste-modifying activity was recently revealed through the construction of a neoculin variant in which all of these residues were replaced with Ala (*12*).

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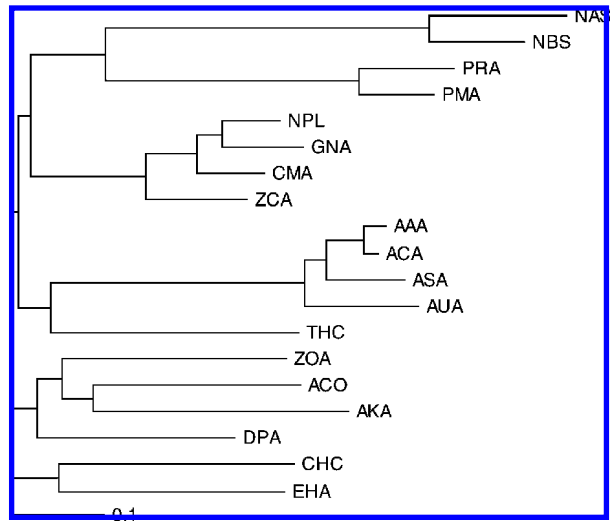

Figure 1. Phylogenetic analysis of the two neoculin subunits and the monocot mannose-binding lectins. The tree was generated using a neighbor-joining method. The lectin sequences are shown with abbreviated names. The sequences of ASA, GNA, and NPL are extracted from Protein Data Bank entries 1BWU, 1MSA, and 1NPL, respectively. Species of origin and GenBank accession numbers for the other sequences used are as follows: PRA, *Polygonatum roseum* agglutinin (AY899824); PMA, *Polygonatum multiflorum* (Solomon's seal) agglutinin (U44775); CMA, *Clivia miniata* (kaffir lily) agglutinin (L16512); ZCA, *Zephyranthes candida* agglutinin (AF527385); AAA, *Allium ascalonicum* (shallot) agglutinin (L12172); ACA, *Alllium cepa* (onion) agglutinin (AY376826); AUA, *Allium ursinum* (ramson) agglutinin (U68531); THC, *Tulipa* hybrid cultivar lectin (U23043); ZOA, *Zingiber officinale* (ginger) agglutinin (AY657021); ACO, *Ananas comosus* (pineapple) lectin (AY098512); AKA, *Amorphophallus konjac* agglutinin (AY191004); DPA, *Dioscorea polystachya* (yam tuber) agglutinin (AB178475); CHC, *Cymbidium* hybrid cultivar agglutinin (U02516); EHA, *Epipactis helleborine* agglutinin (U02515). The bar indicates 0.1 amino acid substitution per site.

For the practical use of neoculin as a sweetener, its safety is a major concern. Neoculin is a heterodimeric protein that consists of an acidic, glycosylated subunit (neoculin acidic subunit, NAS) and a basic nonglycosylated subunit (neoculin basic subunit, NBS). Both share 77% amino acid identity, exhibiting high degrees of amino acid sequence similarity to monocot mannose-binding lectins such as snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), garlic lectin (*Allium sativum* aggulutinin, ASA), and daffodil lectin (*Narcissus pseudonarcissus* lectin, NPL) with about 40−50% amino acid identity (*8, 13*). Plant lectins, also called agglutinins or hemagglutinins, are quite diverse families of carbohydrate-binding proteins. They can have high specificity to and high binding affinity for a particular carbohydrate moiety on the cell surface, thus being used as research tools in the fields of biochemistry, cell biology, and immunology (*14, 15*). Present in common foods, lectins are also regarded as having potential in the diagnosis, prevention, and treatment of chronic diseases, such as cancer (*15*). Furthermore, some of the lectins such as ricin and a kidney bean lectin are known to be toxic to mammals including humans (*16–19*). Ricin in *Ricinus communis*, consisting of two polypeptide chains (A- and B-chains), is highly toxic because of its A-chain, which inhibits the cellular protein synthesis by irreversibly inactivating eukaryotic ribosomes, whereas the B-chain, which is a lectin subunit, serves to attach the toxic subunit to the cell surface (*18, 20, 21*). Meanwhile, the lectin in kidney bean (*Phaseolus vulgaris* agglutinin, PHA) is mildly toxic by virtue of the lectin binding to the cell surface and interfering with the cell membrane transport or signaling, which is the usual cause of lectin toxicity (*16, 17, 19*). GNA, a mannose-binding lectin with high homology to neoculin in amino acid sequence, is also toxic to several species of insect pests but can be regarded as nontoxic to mammals due to the scarcity of α-1,3 terminal mannose residues in their small-intestinal brush border membranes and is often used in experimentations to produce genetically modified crops for a pesticidal purpose (*22*).

With respect to structure, the similarity between neoculin and mannose-binding lectins in terms of primary structure strongly suggests a common architecture in their tertiary structures; our X-ray crystallographic analysis has revealed that a whole structure of neoculin is quite similar to those of the mannose-binding lectins (*23*). Given the strong similarities between neoculin and the lectins in primary and tertiary structure, we investigated those by structural analysis, agglutinin activity assay, viability assay of Caco-2 cells, and DNA microarray to compare their biochemical and genomic properties.

MATERIALS AND METHODS

Primary Structure Analysis. The primary structures of neoculin subunits and monocot mannose-binding lectins were compared by sequence alignment and by generating a phylogenic tree. Proteins with high similarity to NAS and NBS were searched for using FASTA (*24*). In cases when there was little information about signal peptide, the cleavage site was predicted with the program SignalIP 3.0 (*25*) and the sequence of mature proteins used in the analysis. The sequence alignment and phylogenic tree were generated by the program CLUSTALW 1.83 (*26*).

Tertiary Structure Analysis. The coordinate file of the neoculin structure deposited in the Protein Data Bank (PDB entry 2D04) and those of the monocot mannose-binding lectins for which crystal structures are solved, ASA, NPL, and GNA (PDB entries 1BWU, 1NPL, and 1MSA, respectively) were used for the analysis. Structural comparisons between neoculin and the lectins were performed using the programs O (*27*) and LSQMAN (*28*).

Hemagglutination Assay. Dulbecco's phosphate-buffered saline(+) with Ca and Mg was used and termed PBS(+). Heparinized rabbit blood was centrifuged, and erythrocytes were washed three times with PBS(+) and resuspended to 5% (v/v). To 25 μL of 2-fold serially diluted lectin or neoculin solution in V-well microtiter plates was added 25 μL of erythrocyte suspension, and agglutination was scored after 1 h. The hemagglutination assay was also performed with human A, B, and O erythrocytes using the same method to analyze the hemagglutination activity of neoculin and GNA. PHA-

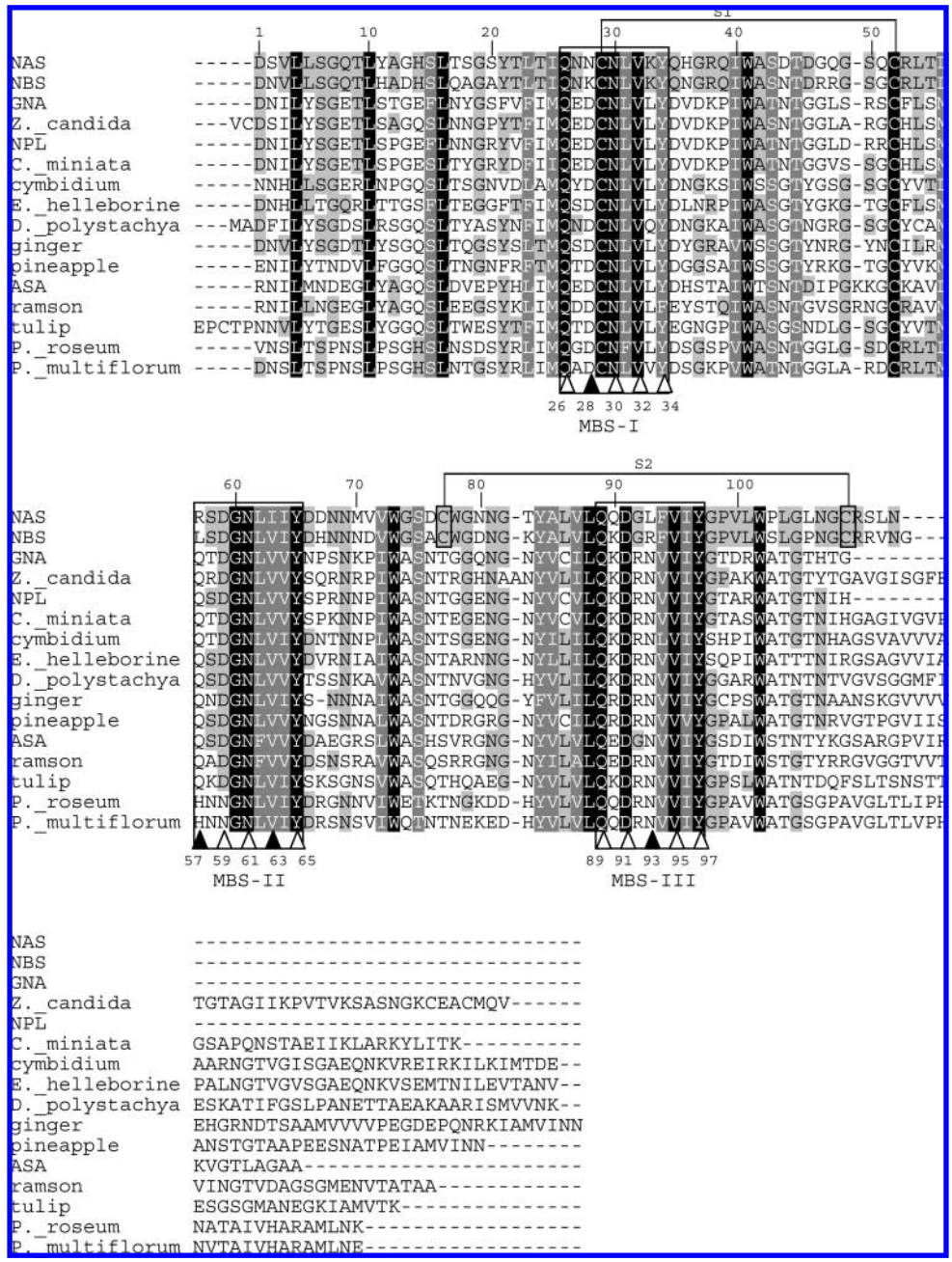

Figure 2. Sequence alignment of neoculin and monocot mannose-binding lectins. The residues completely conserved in all of the molecules are shown as white letters in black boxes. Those well conserved are shown as white letters in dark gray boxes. The residues conserved between NAS and NBS are shown as black letters in light gray boxes. The conserved residues in the mannose-binding sites of the lectins are indicated with arrow heads and residue numbers. The residues in these conserved sites that are substituted in NAS and/or NBS are shown with black arrow heads. The three mannose-binding regions, MBS-I, MBS-II, and MBS-III, are indicated as boxes. The disulfide bond conserved in all of the molecules is shown as S1, and the disulfide bond observed only in neoculin is indicated as S2.

E4 (J-Oil Mills Inc., Tokyo, Japan) was used as positive control in these experiments. The study was approved by the ethics committees of the University of Tokyo.

Cytotoxicity Assay. Caco-2 cells were purchased from the American Tissue Culture Collection (Rockville, MD). PHA-E was purchased from Sigma Chemical Co. The cells were cultivated in 24-well plates (NUNC, Rochester, NY) using Dulbecco's modified Eagle medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 4 mM L-glutamine (MP Biochemical, Inc.), 1% nonessential amino acids (GIBCO, New York, NY), 40 units/mL of penicillin, and 40 μg/mL streptomycin (GIBCO). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded at a density of 1.0×10^5 cells/cm^2. For all of the experiments, Caco-2 cells were cultured for 15 days after reaching confluence. Caco-2 cells were exposed for 24 h to different concentrations of recombinant neoculin. Control cells were fed culture medium containing 1% sterile water. Both control and treated cells were trypsinized and stained with trypan blue (0.4%), and the number of viable (uncolored) cells was counted. Results are represented as mean values of three independent experiments.

DNA Microarray Analysis. Total RNA was prepared from the Caco-2 cells using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan) and purified with an RNeasy mini kit (Qiagen K.K., Tokyo, Japan). The quality and quantity of total RNA were determined by agarose gel electrophoresis and spectrophotometry, respectively. DNA microarray analysis was performed as described previously (*29*). In brief, cDNA was synthesized from 2 μg of purified total RNA, and then

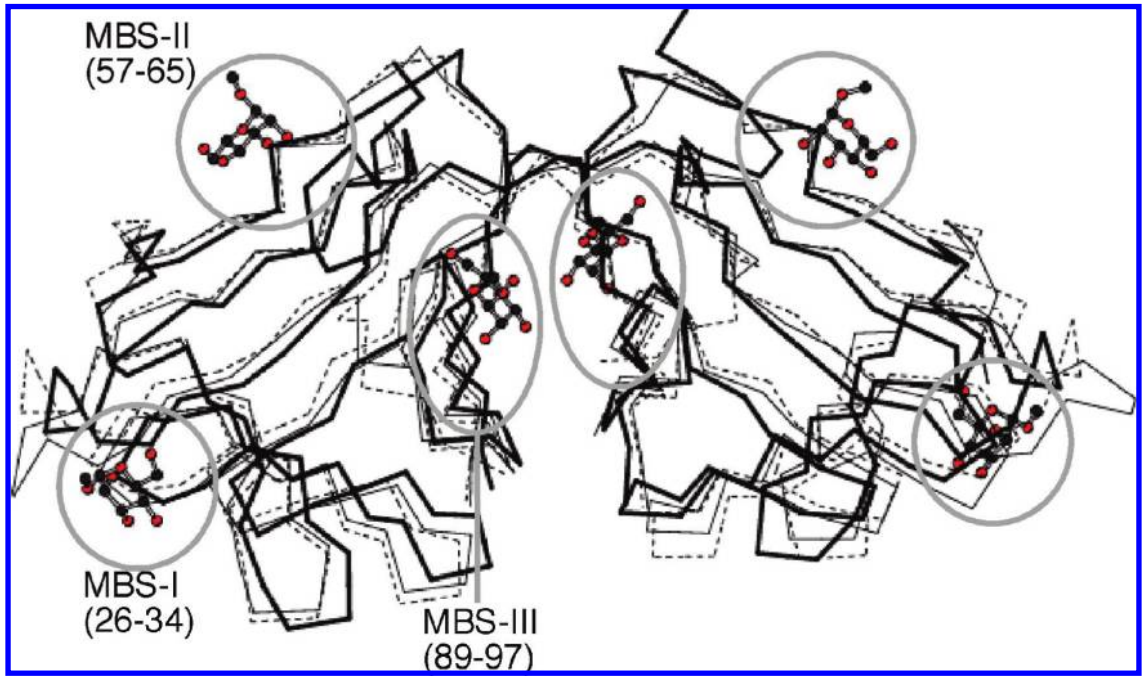

Figure 3. Superposition of neoculin (bold solid line), ASA (thin solid line), and AD dimer of tetrameric GNA (broken line). MeMan molecules bound to GNA are shown as ball-and-stick models. Mannose-binding sites (MBS) are indicated with gray circles.

Table 1. Rmsds upon Superposition of Cα Atoms of the Nine Residues in the Mannose-Binding Motif

	minimum (Å)	maximum (Å)	mean (Å)
Comparison between Neoculin Molecules			
all dimers	0.668	0.898	0.794
all subunits	0.463	0.887	0.667
NAS	0.506	0.680	0.599
NBS	0.463	0.807	0.658
all mannose-binding motifs	0.150	0.700	0.400
first motif (26−34)	0.200	0.590	0.370
second motif (57−65)	0.170	0.400	0.290
third motif (89−97)	0.150	0.370	0.290
Comparison between GNA Molecules			
between the two dimers			0.318
all subunits	0.205	0.372	0.286
all motifs	0.040	0.270	0.160
first motif (26−34)	0.050	0.080	0.070
second motif (57−65)	0.060	0.150	0.110
third motif (89−97)	0.040	0.060	0.050
Comparison between Neoculin and GNA			
all dimers	1.411	1.748	1.581
between NAS and GNA subunit	0.761	0.916	0.844
between NBS and GNA subunit	0.932	1.109	1.030
all motifs	0.160	0.630	0.380
first motif (26−34)	0.160	0.520	0.350
second motif (57−65)	0.320	0.570	0.460
third motif (89−97)	0.190	0.420	0.300

biotinylated cRNA was transcribed using T7 RNA polymerase, fragmented, and added to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA), which contains probes for over 54000 human genes and their fragments. Following hybridization at 45 °C for 16 h, the array was washed and labeled with phycoerythrin. Fluorescence signals were scanned using the Affymetrix GeneChip System.

DNA Microarray Data Analysis. Affymetrix GCOS software was used to reduce array images to the intensity of each probe (CEL files). The CEL files were quantified with the DFW algorithm using the statistical language R (http://www.r-project.org/) and Bioconductor (http://www.bioconductor.org/). Then hierarchical clustering was performed by the pvclust() function in R.

RESULTS AND DISCUSSION

Primary Sequence Analysis. A homology search by FASTA revealed that both NAS and NBS were highly homologous to monocot mannose-binding lectins as shown by their molecular phylogenic tree (**Figure 1**). The alignment of neoculin and monocot mannose-binding lectins (**Figure 2**) showed that there was significant similarity between the neoculin subunits and the lectins. Each lectin molecule possesses three mannose-binding motifs per subunit containing a consensus sequence QXDX-NXVXY that includes five conserved amino acid residues (*30*). Neoculin subunits have corresponding regions similar to this consensus sequence, but there is at least one amino acid substitution in each region (**Figure 2**). In the first pseudo-mannose-binding region from Gln26 to Tyr34, the particular residue, Asp28, conserved in the lectins is replaced with Asn in NAS and with Lys in NBS. In this region, the residue at position 33 is occupied usually by a hydrophobic residue in the lectins (*30*), but by Lys in both NAS and NBS. In the second region from amino acid 57 to 65, position 57, which is usually occupied by Gln or His in the lectins, is replaced with Arg in NAS and with Leu in NBS. In the third region from Gln89 to Tyr97, the residue Asn93 conserved in the lectins is replaced with Leu in NAS and with Arg in NBS.

Tertiary Structure Comparison. Crystal structures of neoculin and two monocot mannose-binding lectins, ASA and GNA, are superimposed in **Figure 3**. This figure shows that the overall dimer structures of these molecules are quite similar. The tertiary structures of neoculin and GNA are compared at dimer, monomer, and mannose-binding site levels, and the rmsd values are summarized in **Table 1**. When compared on a dimer basis, the rmsd values of the four crystallographically independent neoculin dimers range from 0.668 to 0.898 Å, whereas the rmsd between two GNA dimers is 0.318 Å. These values suggest that the neoculin molecule can be more variable in its dimer structure than GNA. The rmsd values between neoculin dimers and GNA dimers range from 1.411 to 1.748 Å.

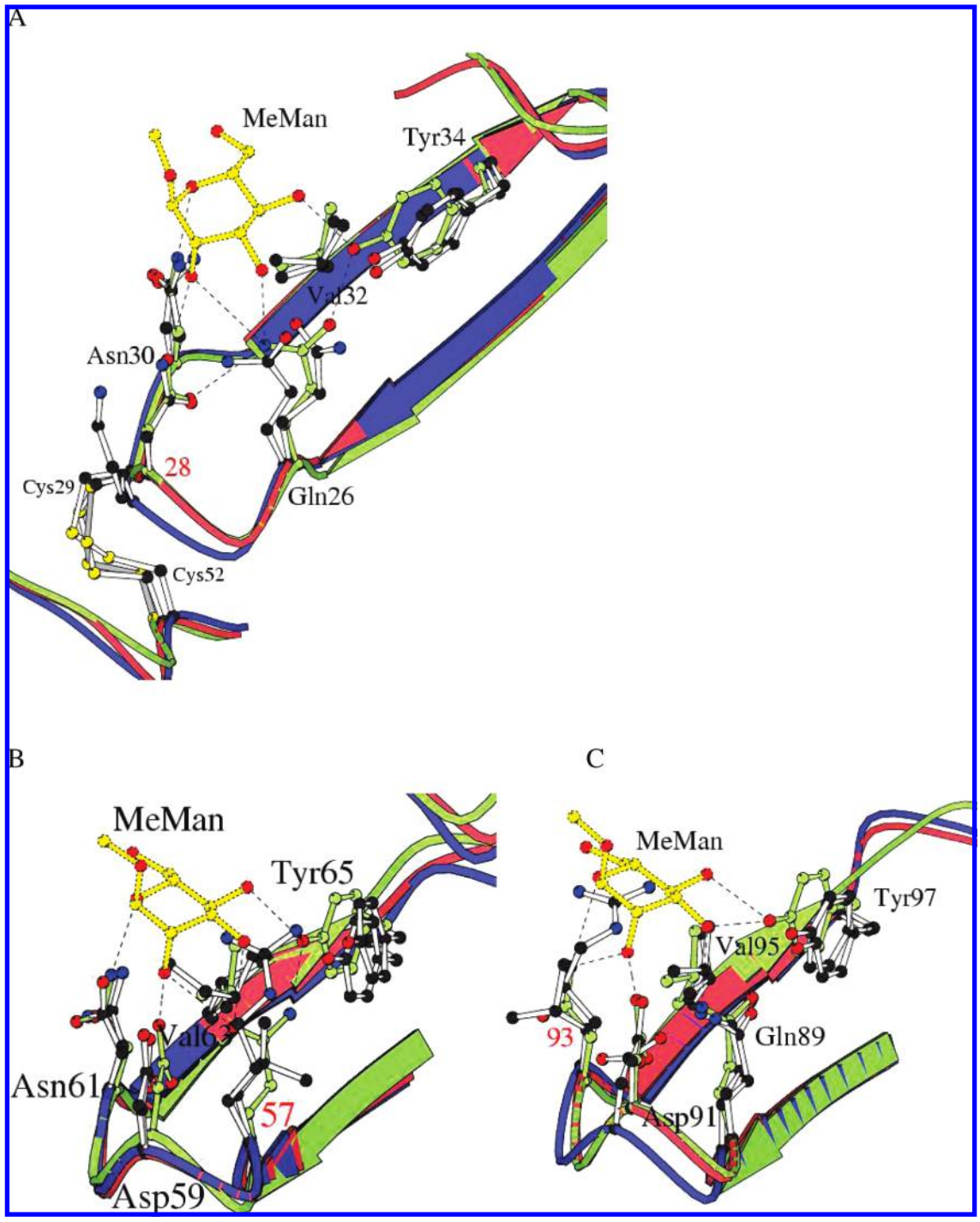

Figure 4. Structural comparison between neoculin and GNA in terms of monocot mannose-binding sites: (**A**) mannose-binding site I; (**B**) mannose-binding site II; (**C**) mannose-binding site III. The main chains of the proteins are drawn as ribbon models, and the side chains of proteins are shown as ball-and-stick models. The structures of MeMan molecules bound to GNA are also shown as ball-and-stick models as yellow dotted lines. The main-chain structure and side chains of GNA are shown in light green, the main-chain structure of NAS is in red, and that of NBS is in blue.

As mentioned above, there are three mannose-binding sites per lectin subunit (**Figure3**); the superpositions of each region in neoculin and GNA (**Figure 4**) demonstrate that the backbone structures of these regions in neoculin and GNA are similar. From the crystal structure of GNA, rmsd values from superposition of the Cα atoms of the nine residues in the motif are in the range of 0.040−0.270 Å for all 12 unique mannose-binidng motif structures. This indicates that the main-chain conformations of these regions are strictly conserved (**Table 1**). In the garlic lectin as well, the rmsd values in these regions are small, in the range of 0.1−0.3 Å (*30*). In the case of the neoculin structure, the rmsd values range from 0.150 to 0.700 Å for all 24 motifs (**Table 1**). The higher rmsd values in the neoculin substructure suggest possible structural uncertainty and/or a lack of rigidity in the pseudo-mannose-binding regions compared to the mannose-binding sites of GNA and ASA. **Figure 4** also shows that the amino acid substitutions which are found in the neoculin subunits can cause a lack of mannose-binding ability mainly by steric hindrance or by the loss of specific interaction between protein and mannose molecules. In the structure of GNA complexed with methyl-α-D-mamnose (MeMan), the side chain of Asp28 is involved in the formation of a hydrogen-bonding network in mannose-binding site I. Asp28 of GNA forms two

Table 2. Hemagglutination Activity of Neoculin and Lectins against Rabbit Erythrocytes and Human Erythrocytes of Different Blood Groups[a]

	minimum concentration (μg/mL)		
erythrocyte	neoculin	GNA	PHA-E4
rabbit	>1000	0.78	nd
human A	>2000	>2000	1.95
human B	>2000	>2000	1.95
human O	>2000	>2000	3.91

[a] Data are given as minimum concentration that showed hemagglutination activity. nd, not determined.

Table 3. Viability of Caco-2 Cells Treated with Various Concentrations of Neoculin[a]

neoculin (mg/mL)	relative viability (%)
0	100.0 ± 2.0
0.03	93.2 ± 1.0
0.1	89.6 ± 2.7
0.3	91.6 ± 2.7

[a] The cells were cultured for 24 h after the addition of neoculin. Values represent means ± SEM (n = 3).

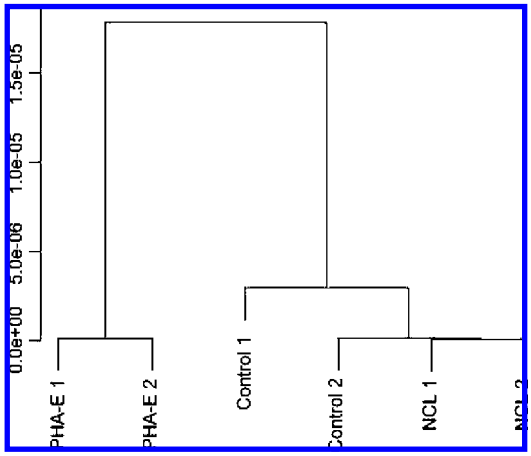

Figure 5. Hierarchical clustering dendrogram of microarray data obtained from lectin-treated or untreated Caco-2 cells. Differentiated Caco-2 cells were treated for 24 h with neoculin (NCL) or PHA-E at 0 mg/mL (control) or 0.03 mg/mL. After treatment, total RNA was extracted and subjected to DNA microarray assay (see Materials and Methods). The vertical scale corresponds to the distance between clusters.

hydrogen bonds between Asp28 Oδ2 and an O atom of MeMan and between Asp28 Oδ1 and Gln26 Nϵ2. However, position 28 in NAS is occupied by Asn and that in NBS by Lys. Thus, the hydrogen-bonding network observed in GNA is destabilized in both NAS and NBS (**Figure 4A**). In mannose-binding site II of GNA, Gln57 is hydrogen-bonded to an O atom of MeMan. This position is occupied by Arg in NAS, which causes steric conflict with mannose. The same position is occupied by Leu in NBS, which also causes the loss of the hydrogen bond between the residue and mannose molecule (**Figure 4B**). The side chain of Asn93 is hydrogen-bonded to MeMan in mannose-binding site III of GNA, but the presence of Leu and Arg at position 93 in NAS and NBS, respectively, results in steric conflicts with the mannose molecule (**Figure 4C**). It has been reported that the backbone of the substructure along with appropriate positioning of the side chains provides the required topology and geometry at the site for mannose binding in the mannose-binding lectins (*30*). Thus, the lack of structural exactitude in the main chain together with the substitutions of the side chains in the mannose-binding sites is apparently a reason for the loss of mannose-binding ability in neoculin.

Hemagglutination Activity. We performed hemagglutination assays using erythrocytes from both humans and rabbits (**Table 2**) to investigate whether neoculin has lectin-like hemagglutination activity. The results of the hemagglutination assay with human erythrocytes revealed that neither neoculin nor GNA had hemagglutination activity at a concentration of 2.0 mg/mL. PHA-E4 used as a positive control showed strong activity at a concentration of 1.95−3.91 μg/mL. With rabbit erythrocytes, neoculin had no activity, whereas GNA agglutinated cells at a concentration of 0.78 μg/mL.

Cytotoxicity Assay and DNA Microarray Analysis. Because some plant lectins including PHA-E are toxic to human small intestines, we tried to verify their safety through observing the effects on differentiated Caco-2 cells, which have long been used as a model for human small-intestinal epithelial cells (*31, 32*).

The effect of neoculin on the viability of differentiated Caco-2 cells was evaluated using a dye exclusion assay. Neoculin was added at 0.03, 0.1, and 0.3 mg/mL, and the cells were cultured for 24 h, with the results shown in **Table 3**. At the three different concentrations of neoculin the relative viability remained at 89.6% or more throughout the assays. A similar tendency was observed when the culture was continued for 48 h, with >98% of the cells surviving in the presence of neoculin. A similar level of survival was observed with GNA, whereas in the presence of PHA-E, a marked reduction in viable cells was observed with relative viabilities of 33% at 0.03 mg/mL and 15% at 0.3 mg/mL (Supporting Information Table S1). These results indicate that neoculin does not exhibit any significant toxicity to Caco-2 cells, unlike PHA-E. We are now preparing further experiments to investigate the effect of neoculin on animals.

We also used DNA microarray techniques to analyze the changes in gene expression profiles upon addition of neoculin to Caco-2 cells. Two independent samples of mRNA were extracted from each of the three Caco-2 cell groups treated with neoculin, PHA-E and water (control), respectively, and these were subjected to DNA microarray analysis. The raw data were quantified using the Distribution Free Weighted method (DFW) (*33*), and then a hierarchical clustering analysis was performed (**Figure 5**). The distance between the neoculin-treated group and the control group was very small, and these clusters were not clearly separated in each sample. In contrast, the cluster of the PHA-E-treated group was formed at a long distance from those of the neoculin-treated and control groups. These results indicate that neoculin has little, if any, effect on the gene expression in Caco-2 cells, whereas PHA-E exerts substantial effect.

Our study has revealed that neoculin has no observable hemagglutination activity. This was confirmed by a structural comparison between neoculin and the monocot mannose-binding lectins. Neoculin showed no significant toxicity to Caco-2 cells with little influence on gene expression, whereas PHA-E, a representative lectin toxic to mammals, showed a significant effect on the viability and gene expression profile of Caco-2 cells. All of these data strongly suggest neoculin to be safe in mammals, encouraging its practical use in the near future.

ABBREVIATIONS USED

ASA, *Allium sativum* agglutinin (garlic lectin); GNA, *Galanthus nivalis* agglutinin (snowdrop lectin); MeMan, methyl-α-D-mannose; NAS, neoculin acidic subunit; NBS, neoculin basic subunit; NPL, *Narcissus pseudonarcissus* (daffodil) lectin; PHA-E, *Phaseolus vulgaris* agglutinin (lectin from kidney bean) E-form.

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Supporting Information Available: Viability of Caco-2 cells treated with various concentrations of neoculin, PHA-E, peanut lectin (PNA), and GNA ($n = 2$). This material is available free of charge via the Internet at http://pubs.acs.org.

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