Galectin LEC-6 Interacts with Glycoprotein F57F4.4 to Cooperatively Regulate the Growth of *Caenorhabditis elegans*

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To study the endogenous counterpart of LEC-6, a major galectin in *Caenorhabditis elegans*, the proteomic analysis of glycoproteins captured by an immobilized LEC-6 column was performed using the nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique. A protein recovered in a significant amount was determined to be either F57F4.3 or F57F4.4, although the method used could not determine which protein was the actual counterpart. Because the knockdown of the F57F4.3/4 genes in *C. elegans* is reported to cause growth retardation, we performed a double knockdown of the *lec-6* and F57F4.3/4 genes. Although the RNA-mediated interference (RNAi) of *lec-6* led to no obvious phenotype, the RNAi of both the *lec-6* and F57F4.3/4 genes led to a significant reduction in growth rate when compared to the RNAi of F57F4.3/4 alone. Furthermore, to clarify which protein, F57F4.3 or F57F4.4, was responsible for the retarded growth, the levels of the F57F4.3/4 proteins expressed in a *C. elegans* wild type and a mutant lacking part of the F57F4.3 gene were compared. The levels of protein expressed by the wild type and the mutant were not significantly different, suggesting that the F57F4.3 protein contributes very little to growth retardation and that the major glycoprotein that interacts with LEC-6 is the F57F4.4 protein. These results suggest that binding with LEC-6 supports the function of F57F4.4 and that their cooperative functioning regulates the growth of *C. elegans*.

Key words   LEC-6; F57F4.4; *Caenorhabditis elegans*; galectin

Galectins are a family of β-galactoside-binding proteins distributed in animals and fungi.[1,2] In the genome of *Caenorhabditis elegans*, more than 10 putative galectin genes have been assigned.[3—6] Galectins are thought to participate in various cellular processes *via* the recognition of the β-galactosides present in a variety of glycoconjugates. The sugar-binding specificities of most galectins have been intensively studied.[5—7] Recently, we reported that the disaccharide unit recognized by the *C. elegans* galectin LEC-6 is Galβ1-4Fuc,[8,9] in contrast to Galβ1-4GlcNAc, which is the corresponding unit recognized by vertebrate galectins. The presence of the Galβ1-4Fuc structure in vertebrates has not yet been reported, nor has the Galβ1-4GlcNAc structure been confirmed in nematodes. This suggests that the co-evolution of galectin binding sites and their sugar structure epitopes occurred independently both in nematodes (protostomes) and vertebrates (deuterostomes), in order to conserve the interaction between galectins and their endogenous counterpart glycoconjugates. However, only a limited number of obvious abnormalities have been observed after the deletion or knockdown of *C. elegans* galectin genes, likely because of compensation by other family members.[10,11] This has made it difficult to understand the overall role of galectins.

LEC-6 is one of the most intensely studied lectins in *C. elegans*, and various glycoproteins and glycans have been assigned to it as potential counterparts.[8,9,12—15] LEC-6 mRNA is expressed mainly in the intestine, according to the Nematode Expression Pattern DataBase (NEXT DB; http://nematode.lab.nig.ac.jp/), and its expression is upregulated by microbial infection.[16] Because the intestine is one of the major organs susceptible to microbial infection,[17] LEC-6 may function as a defense against microorganisms. To attain a more profound understanding of the biological function of LEC-6, more detailed studies of its counterpart glycoconju-

gates are necessary. In this report, we have assigned the protein product of the gene F57F4.4 as an endogenous counterpart to LEC-6 and examined its potential role in the regulation of *C. elegans* growth.

MATERIALS AND METHODS

Materials   The following strains of *C. elegans* were used in this study: Bristol N2 (wild type) and RB2016 (a mutant having a partial deletion in the F57F4.3 gene, provided by the *Caenorhabditis* Genetics Center, National Center for Research Resources of the National Institutes of Health).

Isolation of Glycoproteins Interacting with LEC-6 Preparation of an immobilized LEC-6 column and isolation of LEC-6-binding glycoproteins were performed as previously described.[8] In brief, mixed stage *C. elegans* strain N2 extract was applied to an immobilized LEC-6 column (bed volume, 8 ml; 8.7 mg protein/ml gel). After washing the column extensively with Tris-buffered saline-ethylenediaminetetraacetic acid (TBS-EDTA) (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA; pH 7.5), the adsorbed materials were eluted with TBS-EDTA containing 0.1 M lactose.

Assignment of LEC-6-Binding Glycoproteins by Nano Liquid Chromatography-Tandem Mass Spectrometry Analysis A portion of Fr. 16 (Fig. 1), containing glycoproteins captured by LEC-6, was subjected to ethanol precipitation. The precipitate was dissolved in 6 M guanidine–HCl and 300 mM Tris–HCl (pH 8.5). The proteins were then reduced by the addition of 5 mM dithiothreitol, forming free sulfhydryl groups that were alkylated with 10 mM iodoacetamide. The *S*-carboxymethylated proteins were precipitated with ethanol and subjected to digestion with trypsin, using Trypsin Spin Columns (SIGMA, St. Louis, MO, U.S.A.) according to the manufacturer's instruction. The resulting pep-
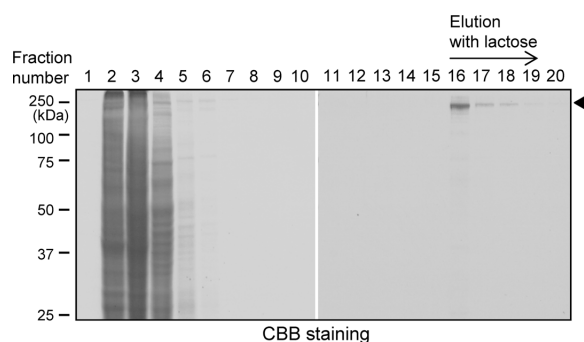
Fig. 1. Affinity Capture of LEC-6-Interacting Proteins from *C. elegans*

C. elegans extract was applied to an immobilized LEC-6 column. Following extensive washing, the bound materials were eluted with 0.1 M lactose. Each fraction was subjected to SDS-PAGE and stained using Coomassie brilliant blue. The numbers on the left of the panel are the molecular masses of standard proteins. The major band, which was identified as F57F4.3/4, is indicated by the arrow.

tides were eluted from the column with water, after which acetonitrile (ACN) and trifluoroacetic acid (TFA) were added to the solution, yielding final concentrations of 2% and 0.1%, respectively. The peptide solution thus obtained was subjected to nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The MS/MS experiment was performed using a LC-MS system consisting of a nanoLC system (Ultimate 3000; Dionex, Sunnyvale, CA, U.S.A.) and an electrospray ionization (ESI)-IT MS system (LXQ; Thermo Fisher Scientific, Waltham, MA, U.S.A.). For each experiment, a 5-μl sample was loaded by an auto sampler onto a C18 trap column (300 μm $\times$ 5 mm; Dionex) quilibrated with 2% ACN, 0.1% TFA at a flow rate of 25 μl/min for desalting and concentration. The trapped peptides were then eluted and separated by a nano capillary column (75 μm $\times$ 15 cm; Nikkyo Technos, Tokyo, Japan). The flow rate was 300 nl/min, beginning with 95% solvent A (5% ACN, 0.1% formic acid) and 5% solvent B (95% ACN, 0.08% formic acid). The percentage of solvent B was gradually increased to 45% from 5 to 45 min. MS and MS/MS analysis were performed using a heated capillary to ionize the peptides (200 °C) at an ESI voltage of 2.0 kV and a collision energy setting of 35%. The 5 most intense product ions from the MS analysis were subjected to MS/MS analysis. The MS/MS spectra obtained were searched against the *C. elegans* protein database downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) using the Proteome Discoverer 1.0 (Thermo Fisher Scientific).

RNA Interference and Growth Rate Analysis For construction of the plasmids used for RNA interference (RNAi) experiments, the *lec-6* gene was amplified by polymerase chain reaction (PCR) using the primers, 5′-GAGAATCAAC-CATGATCGGAGGAG-3′ and 5′-AAAACCAAGGTTTATT-GATATGAAAT-3′. A section of the F57F4.3 gene, which is almost 100% identical to F57F4.4, was also amplified using the primers, 5′-AGGATGAGTTGTGTAACAAGGTC-3′ and 5′-CAGGTGTAGAGAGTAGCGTAGT-3′. The amplified fragments were inserted into L4440 vectors. The *Escherichia coli* strain HT115 (DE3) was transformed with these plasmids. As a control, *E. coli* were also transformed using empty L4440 vectors. Worms were fed as previously described,[18] with the exception that the IPTG concentration was 0.4 mM. Feeding was begun at the L4 stage. After 1 d,

adult worms were transferred to new plates containing the transformed *E. coli* and were allowed to lay eggs for 2 h at 25 °C. Adult worms were then removed, and the F1 eggs were cultured for 3 d at 25 °C. The number of worms that reached the L4, or adult, stage was scored ($n > 100$). The efficacy of the knockdown of gene expression was verified by reverse transcription (RT)-PCR: based on the band intensity, the amount of the mRNA was decreased to about 20% of the control.

Preparation of Anti-F57F4.3/4 Rabbit Antiserum A section of the F57F4.3 open reading frame containing the *Bam*HI and *Not*I sites was amplified by PCR. This region corresponds to amino acid residues 1009—1575 and is almost 100% identical to that of F57F4.4. The primers 5′-GGATCCATCACCTGCCCAGCTGGTC-3′ and 5′-GCGG-CCGCTCCACCATATCCAGCCAAATC-3′ were used as forward and reverse primers, respectively. To generate the plasmid for expression in *E. coli*, the PCR fragment was subcloned into the *Bam*HI and *Not*I sites of the pET-21a vector (Merck, Darmstadt, Germany).

The recombinant His-tagged F57F4.3 (1009—1575) protein was expressed in *E. coli*. Because the recombinant protein was nearly insoluble, the inclusion body was collected as previously described.[19] The His-tagged F57F4.3 (1009—1575) protein was recovered from the inclusion body using TALON® Metal affinity resin (Takara Bio, Shiga, Japan) and eluted with imidazole according to the manufacturer's recommendation. The fraction containing purified protein was subjected to trichloroacetic acid (TCA) precipitation to remove the imidazole and concentrate the protein. The protein was then dissolved in a buffer containing 4 M Urea and used as an antigen. Rabbit immunization and preparation of anti-F57F4.3/4 antiserum were performed at Keari (Osaka, Japan).

Western Blotting Mixed stage *C. elegans* N2 (Wild type) and RB2016 (F57F4.3 gene partially deleted) strains were suspended in a sodium dodecyl sulfate (SDS)-sample buffer (50 mM Tris–HCl, pH 6.8; 1% SDS; 8% glycerol; 0.01% pyronin-Y; and 2% 2-mercaptoethanol) and disrupted by sonication. After centrifugation, the supernatant was used as the worm extract. The extracts of *C. elegans* N2 and RB2016 strains were subjected to western blotting using anti-F57F4.3/4 antiserum as a probe, as previously described.[19]

RESULTS AND DISCUSSION

Isolation and Identification of Proteins Bound by LEC-6 To capture counterpart glycoproteins of LEC-6, we applied *C. elegans* extract to an immobilized LEC-6 column, after which the adsorbed materials were released by addition of lactose (Fig. 1). Fraction 16 of the column eluate, containing materials bound by LEC-6, was subjected to ethanol precipitation. The precipitate was then digested by trypsin, and the peptide mixture was analyzed by nanoLC-MS/MS. Table 1 displays the proteins found in the eluate. For most of these proteins, the presence of *N*-glycan(s) recognizable by LEC-6 has been confirmed.[13,14] This table also includes LEC-6 itself and LEC-1, even though these are not glycoproteins. These galectins seem to have been indirectly adsorbed by the immobilized LEC-6 column by attaching to 1 or more glycoproteins, which can have multiple galectin epitopes in a sin-

Table 1. Potential Binding Partners of LEC-6 Identified by NanoLC-MS/MS with a Significant Score

Sequence name	Main name	Unique peptides	Molecular weight (kDa)	Coverage (%)	Score
C37C3.6	MIG-6	25	169.5	27.5	222
K07E12.1	DIG-1	24	1372.5	2.9	203
F40F4.6		17	243.3	10.4	163
F28B4.3		14	244.7	11.6	149
F57F4.3/4		13	227.3	10.9	141
T25C12.3		17	230.7	15.2	141
F55D10.1	AMAN-1	11	109.3	16.2	111
K07H8.6	VIT-6	9	193.1	7.8	101
F09B12.3		11	66.6	24.9	88
C42D8.2	VIT-2	13	187.6	13.6	80
Y55B1AR.1	LEC-6	4	16.0	54.1	63
Y16B4A.2		5	241.0	3.3	51
K06G5.1		4	54.7	10.7	41
R09H10.5		3	175.2	3.5	41
F35C5.8	CLEC-65	5	41.6	19.9	39
H34I24.2		3	47.3	14.4	38
R57.1		5	63.9	12.5	37
W09H1.6	LEC-1	5	31.8	19.7	37
C01B10.6		3	51.5	9.2	35
T01D3.6		3	102.2	5.6	34
R08E3.2		3	35.0	15.9	27
F19C7.1		3	29.4	17.3	27
LLC1.2		5	30.3	34.9	25
M02F4.7	CLEC-265	2	35.9	11.2	25
T02B5.3		2	76.8	6.1	23
F25E2.2		3	69.4	5.8	22
F54F11.2		2	164.2	4.3	21
F21F8.7	ASP-6	2	41.5	8.7	21
C49C8.5		3	48.7	10.0	20
F31E3.5	EFT-3	2	50.6	5.4	20
Y34B4A.9		3	25.3	22.7	17
C49C3.4		2	169.6	1.9	14
T16G12.1		2	216.1	2.1	13

gle molecule.

Next, we isolated the most intense band observed in Fr. 16 (marked by the arrow in Fig. 1) and analyzed it more thoroughly using both nanoLC-MS/MS and N-terminal sequencing (data not shown). The proteins responsible for this band were found to be either F57F4.3, F57F4.4, or both. Since the differences between the F57F4.3 and F57F4.4 genes are very small, it was not immediately possible to discriminate between these 2 candidates.

F57F4.3/4 and LEC-6 Cooperatively Regulate the Growth of *C. elegans* The proteins encoded by F57F4.3/4 were identified as candidates of major LEC-6 glycoprotein counterparts. F57F4.3/4 proteins have 9 *N*-glycosylation sites, which are modified with *N*-glycans recognizable by LEC-6 because the corresponding tryptic peptides were captured by an immobilized LEC-6 column.[13,14] Both LEC-6 and F57F4.3/4 are obviously expressed in the intestine of *C. elegans* at the mRNA level according to the NEXT DB. This suggests an interaction between LEC-6 and a product of F57F4.3/4 within the body of *C. elegans*.

An F57F4.4 mutation was reported to be lethal in worms (WormBase; http://www.wormbase.org/), and the knockdown of the F57F4.3/4 genes resulted in growth retardation.[20] Because LEC-6 may influence the role of F57F4.3/4 proteins, we examined the effect of double-knockdown of the *lec-6* and F57F4.3/4 genes on the growth rate of F1 worms (Fig.
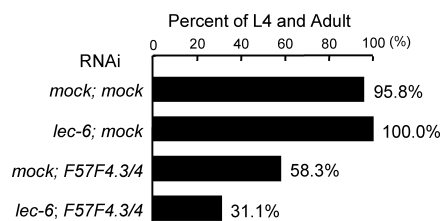

Percent of L4 and Adult

Fig. 2. Effect of the Double-Knockdown of the *lec-6* and F57F4.3/4 Genes on the Growth of *C. elegans*

The worms were fed with equal amount of *E. coli* expressing double-stranded RNA targeted to indicated genes, or mock. Feeding was begun at the L4 stage. After F1 eggs were laid, the proportion of these eggs that reached the L4, or adult, stage in 3 d was scored.

2). The knockdown of *lec-6* alone did not result in any obvious phenotype. However, compared with a knockdown of F57F4.3/4 alone, a double-knockdown of both *lec-6* and F57F4.3/4 increased the proportion of worms that grew slowly. These results imply that LEC-6 supports or enhances the function of the F57F4.3/4 genes, which play a regulatory role in the growth of *C. elegans*.

Identification of F57F4.4 as a Major Glycoprotein Counterpart of LEC-6 Because proteomic analysis failed to identify which protein, F57F4.3 or F57F4.4, was actually expressed in *C. elegans*, we compared the levels of expression for each protein in N2 (wild type) and RB2016 mutant (F57F4.3 partially deleted) strains. No obvious phenotype has been observed in the RB2016 mutant despite its lack of a section of the F57F4.3 gene. The deletion site of this mutant was confirmed by genomic DNA sequencing (data not shown), and the position of this site was found to be 6393677—6394334 on chromosome 5. Thus, the protein encoded by the mutated F57F4.3 gene in the *C. elegans* RB2016 strain should be composed of 1935 amino acid residues, in contrast to the intact protein containing 2153 residues in the N2 strain (Fig. 3A). N2 and RB2016 worm extracts were subjected to Western blot analysis probed with anti-F57F4.3/4 antiserum (Fig. 3B). No significant difference in band pattern was observed between the N2 and RB2016 extracts. Moreover, a protein band corresponding to the molecular weight of 1935 amino acid residues was not observed in the extract of the mutant RB2016 worm. This suggests that F57F4.3 is scarcely expressed in the mutant worm and is likely to be scarce in the wild-type N2 worm.

It has been reported that RNAi of F57F4.3/4 causes slow growth and that a partial deletion of the F57F4.3 gene causes no observable abnormality. However, partial deletion of the F57F4.4 gene is lethal (WormBase). These findings, along with our present results, suggest that the F57F4.3 gene is scarcely expressed and has little-to-no biological function under standard laboratory conditions. Therefore, it is likely that the major endogenous counterpart glycoprotein of LEC-6 is F57F4.4, and they cooperatively regulate the growth of *C. elegans*.

Recently, Maduzia *et al.* observed the interaction between LEC-6 and a F57F4.3/4 proteins *in vivo*, and altered subcellular distribution of the glycoprotein ligands in a LEC-6-deletion mutant worm.[15] Our present results are consistent with, and support to their observation; that is, we also found LEC-6 interacts with F57F4.3/4 protein *in vitro*, and furthermore, presented a evidence that the interaction between these

A.

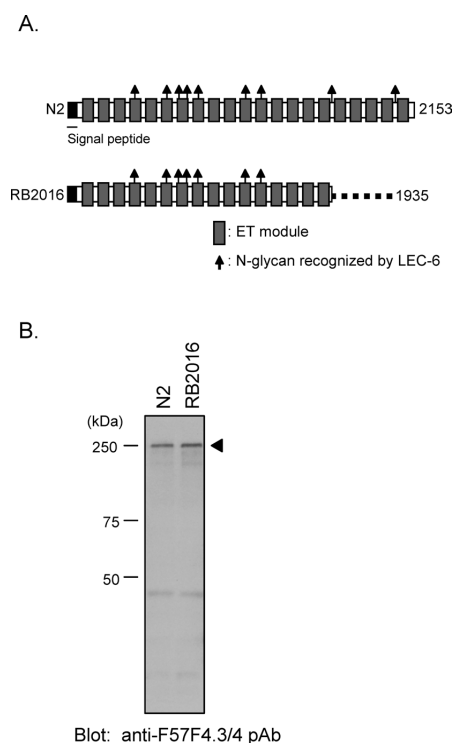

B.

Blot: anti-F57F4.3/4 pAb

Fig. 3. Western Blotting Analysis of the RB2016 Strain Extract

(A) Illustrations of the domain structures of the F57F4.3 protein in N2 and RB2016 worms. Numerals indicate the number of amino acid residues. Gray boxes indicate ET modules (function yet unknown). In RB2016 worms, deletion in the respective gene results in complete different amino acid sequence from the wild type F57F4.3 protein. Broken line indicates the changed primary structure in the C-terminal region (from amino acid residues number 1650). Arrows indicate N-glycosylation sites modified with N-glycans that are recognizable by LEC-6. (B) The extracts of C. elegans N2 (Wild type) and RB2016 (F57F4.3 gene partially deleted) strains were subjected to Western blotting probed with anti-F57F4.3/4 antiserum. The numbers on the left of the panel are the molecular masses of standard proteins. The arrow indicates the position of the F57F4.3/4 proteins.

two proteins *in vivo* has a certain role in growth regulation of *C. elegans*.

Because the development and growth of *C. elegans* is a complicated process that may be affected by various factors, including bacterial infection, the precise function of F57F4.4 in the growth of *C. elegans* remains uncertain. Mutant worms defective in N-glycosylation have been isolated and found to display altered susceptibilities to bacterial pathogens, suggesting the importance of N-glycosylation in host defense systems.[21] The N-glycans present on F57F4.4 should also be missing in mutants defective in N-glycosylation, and would therefore be unable to interact with LEC-6. Combined with the findings that *lec-6* and F57F4.4 gene are up-regulated by bacterial infection[16] also suggesting their role in host defense as well, the interaction between LEC-6 and F57F4.4 has a role to protect worm from microbial infection.

The present study demonstrates that F57F4.4 is one of the functional counterparts of LEC-6. These findings also indicate the importance of the lectin-glycan interaction in nematodes, as is the case in vertebrates. This supports the idea that the importance of the interaction between galectins and their counterparts might be a driving force for their co-evolution.

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