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Enhanced circulating soluble LR11 in patients with coronary organic stenosis

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ABSTRACT

LR11, an LDL receptor family member, is expressed in intimal smooth muscle cells. It was found that the soluble form of LR11 (sLR11) is detected in serum, and the circulating sLR11 levels are positively correlated with intima-media thickness of carotid arteries in dyslipidemic subjects. To clarify the significance of serum sLR11, the circulating sLR11 levels in patients with organic coronary stenosis and the contributing risk factors for them were studied. The subjects, 150 patients with symptoms of coronary artery disease, underwent coronary angiographic examination, and were divided into sex- and age-matched two groups; one is organic coronary stenosis group (OCS) and the other is normal coronary group (NC). Serum sLR11 levels were significantly higher in OCS than in NC (4.9 ± 2.7 U vs 3.6 ± 1.8 U, $p < 0.05$). Multivariate regression analysis showed that circulating sLR11 is independent contributing factor for the OCS, as well as diabetes mellitus and dyslipidemia. Among various coronary risk factors for sLR11 level, HbA1c showed the highest correlation coefficient ($p < 0.01$).

These results suggest that the circulating sLR11 might reflect coronary organic stenosis, and that hyperglycemic condition might be promoting factor for expression of LR11 in intimal smooth muscle cells.

1. Introduction

In the formation of atherosclerosis, migration of vascular smooth muscle cells (SMCs) from the media to the intima is the key step [1,2]. Following migration, SMCs change the phenotype and proliferate in the intima, resulting in intimal thickness. Furthermore, proliferating SMCs secrete matrices and proteases to form atheromatous lesions under the influence of stimulatory cytokines [3–6].

Recently, we [7] identified LR11, which is an LDL receptor family member with poorly defined function, and observed the expressing of LR11 specifically in intimal SMCs, but not in medial SMCs, macrophages or lymphocytes in the arterial wall [7–10]. LR11 as both the membrane-spanning and the shed soluble (sLR11) forms bind to urokinase-type plasminogen activator receptor (uPAR) on the cell surface [8,11]. Over-expression of LR11 in SMCs enhances their migration via elevated levels of uPAR, and appears to thereby increase the activation of the uPA system [7,12].

Interestingly, the sLR11 was detected in the serum, and that the circulating sLR11 levels are positively correlated with intima-media thickness of carotid arteries in dyslipidemic subjects [13]. The relationship of the sLR11 levels in serum with other risk factors for atherosclerosis, such as age, sex, smoking, blood pressures, serum lipids, and plasma glucose was not observed. But, the precise was not clear yet.

It is reported that arterial intimal thickening after balloon catheter injury was enhanced in diabetic animals than control [6,14]. Clinically, increased intimal-medial thickness of carotid artery in type 2 diabetes was reported [15,16]. Thus, a relationship between coronary stenosis and sLR11 level, and also a relationship between sLR11 and diabetic condition were suspected.

In this report, we investigated the significance of circulating sLR11 in organic coronary stenosis (OCS) of the patients with a suspicion of coronary artery diseases (CAD). Contributing factors for the elevation of serum sLR11 were also analyzed.

2. Subject and methods

The subjects were 150 persons who were suspected to have coronary artery disease and who underwent coronary angiography at Toho University Sakura Hospital Cardiovascular Center were

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recruited for the study. Patients suffered from chronic heart disease with ejection fraction < 50% or chronic renal failure with serum creatinine > 1.3 mg/dl were excluded from the study analysis. The study protocol was approved by the Human Investigation Review Committee of Toho University Sakura Hospital, and informed consent was given by each patient.

The angiographical severity of coronary stenosis was assessed in the worst view position, and the percentage of luminal narrowing was recorded according to the American Heart Association reporting system [17]: organic coronary stenosis was defined as a stenosis with ≥75% diameter, and normal coronary artery (NC) was defined as without significant stenosis. Blood sample were collected in the morning after an overnight fast. Lipid variables and fasting blood glucose were measured by standard laboratory techniques. Serum insulin was measured by an enzymatic-immunological assay. Homeostasis model assessment insulin resistance index (HOMA-IR) was defined as: (plasma glucose × serum insulin)/405 [18]. Potential risk factors for atherosclerosis were analyzed, including age, sex, body mass index (BMI), smoking, and histories of hypertension, diabetes mellitus or dyslipidemia. Hypertension was defined as a history of hypertension (systolic pressure > 140 mmHg or diastolic pressure >90 mmHg). Diabetes mellitus was defined as a history of diabetes mellitus having fasting blood glucose > 126 mg/dl and HbA1c > 5.8%. Dyslipidemia was defined as a history of serum total cholesterol >220 mg/dl and/or triglyceride >150 mg/dl in the fasting and/or HDL-cholesterol <40 mg/dl.

3. Measurement of serum sLR11 concentration

Fasting blood samples were collected and were centrifuged immediately after collection to measure serum sLR11 levels. Fifty microliters of serum was purified using a 39 kDa receptor-associated protein (RAP)-GST affinity beads (Cosmo Bio). For immunoblotting, equal amount of protein extracted from pelleted beads was subjected to 10% SDS-PAGE after heating to 95 °C for 5 min as described [13] under reducing conditions, and transferred to a nylon membrane. Incubations were with antibody against LR11 (5-4-30-19-2 at 1:500 dilution) [13], followed by peroxidase-conjugated anti-mouse IgG. Development was performed with the ECL detection reagents (Amersham Pharmacia). The signals were quantified by densitometric scanning using NIH image™ software. The sLR11 levels in each human serum (50 μl) was determined as an averaged value of three quantified signal intensities resulting from independent assays using samples with blind indication, and expressed as a ratio to that of a standard serum. The immunological estimation indicated that the signal of 1 U (in 50 μl serum) corresponded to approximately 50 ng/ml of recombinant sLR11.

4. Statistics

The results are shown as mean ± SD or proportion (%) for each index. Statistical analysis was performed with SPSS version 13.0 (SPSS Japan Inc.). The unpaired *t*-test and the *chi*-square test were used to compare the continuous and the categorized variables, respectively. Pearson's correlation coefficient analysis was used to assess association between measured parameters. Subsequently, multiple linear regression analyses were used to calculate the ORs for the OCS (i) by controlling for all risk factors (age, sex, BMI, smoking, diabetes, hypertension, dyslipidemia and sLR11) (Model 1); (ii) by additionally controlling for BMI, diabetes, dyslipidemia, and sLR11, which are significantly associated with OCS by above analyses (Model 2). These risk factors were scored as explanatory factors, and subordinate variable was OCS = 1 and NC = 0. A value of $p < 0.05$ was considered significant. Multivariate analysis was performed by multiple regression analysis.

Table 1
Characteristics of the normal coronary artery and organic coronary stenosis subjects.

	NC	OCS	*p* value
n	55	95	
Male (%)	65.5	74.7	0.23
Age (y)	66.1 ± 8.4	66.5 ± 9.7	0.87
BMI (kg/m^2)	23.9 ± 3.0	25.1 ± 3.4	<0.05
Diabetes (%)	5.5	33.7	<0.01
Hypertension (%)	56.4	64.2	0.34
Dyslipidemia (%)	52.7	85.3	<0.01
sLR11(U)	3.6 ± 1.8	4.9 ± 2.7	<0.01
Fasting blood sugar (mg/dl)	106.7 ± 13.4	111.5 ± 27.1	0.22
Insulin (μU/dl)	6.2 ± 3.9	7.9 ± 4.9	<0.05
HOMA-IR	1.7 ± 1.1	2.2 ± 1.9	<0.05
Medications			
Administration of statin (%)	18.2	66.3	<0.01
Administration of ACE-I or ARB (%)	29.1	42.1	0.13

Plus–minus values are means ± SD. The unpaired *t*-test was used for continuous variables, and the *chi*-square test was used for categorized variables. BMI: Body mass index. Circulating sLR11 levels in NC group and OCS group were 3.6 ± 1.8 U and 4.9 ± 2.7 U, respectively, indicating that the sLR11 levels in OCS were significantly higher than those in NC ($p < 0.01$).

5. Results

5.1. Circulating sLR11 levels in NCA and OCS groups

The subjects were classified into age- and sex-matched two groups, according to the angiographical evaluation. Normal coronary artery group is composed of 55 subjects and organic coronary stenosis group is composed of 95 subjects (Table 1). BMI, histories of diabetes and dyslipidemia were significantly increased in the OCS group comparing with the NC group. Smoking and the history of hypertension were not different between the two groups. Insulin levels and HOMA-IR levels were significantly increased in the OCS group. Circulating sLR11 levels in NC group and OCS group were 3.6 ± 1.8 U and 4.9 ± 2.7 U, respectively, indicating that the sLR11 levels in OCS were significantly higher than those in NC ($p < 0.01$). Note that we have reported that the mean circulating sLR11 levels in four-hundreds dyslipidemic subjects are 3.0 ± 1.0 U [13]. Thus, circulating sLR11 levels increased in the patients with organic coronary stenosis among the patients taking angiographical examination with a suspicion of coronary arterial diseases.

5.2. Multivariate analysis of sLR11 and other risk factors for OCS

We next analyzed the significance of sLR11 in comparison to other risk factors for OCS in all subjects (Table 2). The multivariate analysis of all variables (Model 1) for OCS showed that circulating sLR11 and the histories of diabetes or dyslipidemia were explanatory factor for OCS independent from other variables. The Model 2 analysis using the limited variables which have been shown to be significantly increased in OCS (see Table 1) showed that circulating sLR11 is still an independent factor for OCS. These results showed that the circulating sLR11 level was enhanced in OCS group among patients with a suspicion of CAD and taking coronary angiography.

5.3. Correlation of serum sLR11 with other various parameters in all subjects

As shown in Table 3, a negative correlation between sLR11 concentration and HDL-cholesterol ($r = -0.161$, $p < 0.05$) and a positive correlation between sLR11 and triglyceride ($r = -0.161$, $p < 0.05$) were found. Furthermore, BMI ($r = 0.182$, $p < 0.05$), insulin (0.186, $p < 0.05$) and HOMA-IR (0.242, $p < 0.01$) and HbA1c ($r = 0.272$, $p < 0.01$) showed significant positive correlations with sLR11, respectively. But there was no significant correlation between cir-

Table 2
Multivariate assessment of the effect of sLR11 and other risk factors on OCS BMI, sLR11 and age were analyzed per 0.1 kg/m^2, 0.1 U, and 10 y increase, respectively. Models 1 and 2 are described in Methods. BMI: Body mass index.

	OR (95% CI)	*p* values
Model 1		
BMI, per 0.1 kg/m^2 increase	1.00 (0.99–1.02)	0.85
Diabetes	7.98 (2.29–27.77)	<0.01
Dyslipidemia	4.03 (1.61–10.08)	<0.01
Male	1.48 (0.54–4.07)	0.44
Hypertension	1.53 (0.67–3.48)	0.31
Smoking	2.14 (0.88–5.21)	0.09
sLR11, per 0.1 U increase	1.03 (1.00–1.05)	<0.01
Age, per 10 y increase	1.15 (0.72–1.82)	0.93
Model 2		
BMI, per 0.1 kg/m^2 increase	1.04 (0.91–1.15)	0.57
Diabetes	6.36 (1.97–20.51)	<0.01
Dyslipidemia	4.55 (1.86–1.61)	<0.01
sLR11, per 0.1 U increase	1.02 (1.00–1.04)	<0.05

The multivariate analysis of all variables (Model 1) for OCS showed that circulating sLR11 and the histories of diabetes or dyslipidemia were explanatory factor for OCS independent from other variables. The Model 2 analysis using the limited variables which have been shown to be significantly increased in OCS (see Table 1) showed that circulating sLR11 is still an independent factor for OCS.

culating sLR11 levels and age, sex, white blood cell, red blood cell, platelet, total cholesterol, LDL-cholesterol, non-HDL-cholesterol or fasting blood sugar.

5.4. Multiple regression analysis for sLR11 in all subjects

Table 4 shows multiple regression analysis for sLR11concentratin. Variables in simple liner regression analysis with $p<0.05$ were included into the multiple regression analysis model. The levels of sLR11 significantly correlated with HDL-cholesterol, triglyceride, HbA1c, BMI and HOMA-IR. Among these variables, only HbA1c concentration showed independent correlation with sLR11 levels (t-value = 3.02 $p<0.01$).

Table 3
Correlation of Serum Soluble Form of LR-11 with various parameters in all subjects.

	Pearsons correlation coefficient	*p* values
Age	0.121	0.14
Male	−0.150	0.07
Total cholesterol (mg/dl)	0.067	0.42
HDL-cholesterol (mg/dl)	−0.160	<0.05
Triglyceride(mg/dl)	0.161	<0.05
LDL-cholesterol(mg/dl)	0.101	0.11
Non-HDL-cholesterol (mg/dl)	0.144	0.08
Fasting blood sugar (mg/dl)	0.077	0.35
Insulin(μU/dl)	0.186	<0.05
HOMA-IR	0.242	<0.01
HbA1c (%)	0.272	<0.01
Body Mass Index (kg/m^2)	0.182	<0.05

A negative correlation between sLR11 concentration and HDL-cholesterol ($r=-0.161$, $p<0.05$) and a positive correlation between sLR11 and triglyceride ($r=-0.161$, $p<0.05$) were found. Furthermore, BMI ($r=0.182$, $p<0.05$) and HbA1c ($r=0.272$, $p<0.01$) showed significant positive correlations with sLR11, respectively.

Table 4
Results of multiple regression analysis for soluble form of LR11 in all subjects.

	Partial regression coefficient (b)	t-value	*p* value
X			
HbA1c (%)	0.21	2.50	<0.01
HOMA-IR	0.11	1.23	0.22
Body Mass Index (kg/m^2)	0.10	1.16	0.25
HDL-cholesterol (mg/dl)	−0.09	−1.05	0.29
Triglyceride (mg/dl)	0.05	0.59	0.56
Y Soluble form of LR 11			

X, explanatory factor; Y, subordinate variables; Correlation coefficient (R) = 0.35 F value = 4.1, $p=0.002$, ($n=150$); The levels of sLR11 significantly correlated with HDL-cholesterol, triglyceride, HbA1c, and BMI. Among these variables, only HbA1c concentration showed independent correlation with sLR11 levels (t-value = 3.02 $p<0.01$).

6. Discussion

Coronary organic stenosis is formed mainly with intimal thickness which is composed of proliferative intimal smooth muscle cells and matrix components accompanying with lipid pool [19–21]. In our cases, sLR11 was higher in OCS group than that of in NCA group. We have recently reported that LR11 is produced by the intimal SMCs, and considerable amounts of the shed sLR11 enhance SMC migration in vitro [13]. Therefore, high sLR11 concentration may reflect the pathophysiological condition of intimal SMCs. And we also reported that sLR11 is a circulating marker for IMT independent from the other classic risk factor for atherosclerosis in dyslipidemic subjects without CAD or diabetes [13]. Considering the facts that LR11 is highly expressed in intimal SMCs, macrophages, or lymphocytes [7–10], the above results strongly suggest that circulating sLR11 level reflect the amount of intimal SMC in coronary arteries. Next, the contributing risk factors for elevation of circulating sLR11 were studied. Although multiple regression analysis showed HbA1c was only significant factor correlated with sLR11, insulin resistance relating factors such as BMI, HOMA index, low HDL-cholesterol, high triglyceride were related with sLR11 in single regression analysis, indicating that sLR11 might be induced with the state of insulin resistance in addition to diabetes mellitus.

The reason why diabetic condition is tightly correlated with sLR11 levels in serum is not available yet, but it has been reported that arterial intimal thickening after balloon catheter injury was enhanced in diabetic animals than controls [6,14]. From these observations, diabetic condition may induce the expression of sLR11 directly, and also modify the phenotype of smooth muscle cell into so-called synthetic type in the arterial wall. Those possibilities are currently under investigation.

In summary, the results obtained from the patients with a suspicion of coronary artery diseases suggested that circulating sLR11 may relate to coronary organic stenosis, and that hyperglycemic condition is a promoting factor for expression of LR11 in vascular SMCs.

6.1. The limitation

The limitation of the present investigation is at first a lack of information about the serum sLR11 data at acute phases of coronary artery diseases. Second, the data may be influenced by the continuous medication. More of subjects have received the treatments against dyslipidemia with statins in OCS group than in NC group, and most of subjects have received against hypertension with angiotensin II receptor type 1 blockers (ARBs) in the present study (Table 1). Considering the facts that statins and ARBs inhibit the sLR11 expression in the cultured SMCs [10,13], the circulating sLR11 levels may be influenced by these treatments in addition to coronary artery diseases. Further studies to investigate the effects of these drugs on the circulating LR11 levels are in progress. Third, HOMA-IR was assessed as a marker of insulin resistance. If this is the case, glucose clamp method might be better than HOMA-IR; but they are problematic in daily practice, and HOMA-IR considered a reliable insulin resistance marker, in vivo, especially in subjects whose fasting blood glucose concentration were not so high. Therefore we used HOMA-IR as a marker of insulin resistance, considering the burden on patient and medical stuff.

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Increased Levels of Soluble LR11 in Cerebrospinal Fluid of Patients with Alzheimer Disease

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Key Words
LR11 · Lipoprotein receptor · Alzheimer disease · Biomarker · ApoE4

Abstract
Background: Recent genetic and pathological studies have suggested that a lipoprotein receptor, LR11, is intricately implicated in the pathogenesis of Alzheimer disease (AD). We have recently established a novel sandwich ELISA, which enabled the sensitive quantification of a soluble LR11 (sLR11). By this ELISA, we attempted to determine the difference in the levels of CSF sLR11 in AD patients. ***Methods:*** We examined CSF from 29 AD patients, 20 frontotemporal lobar degeneration patients and 27 age-matched control subjects. The CSF sLR11 level as well as the levels of tau and β-amyloid42 (Aβ42) were determined by sandwich ELISA. ***Results:*** The CSF tau level and tau/Aβ42 ratio were significantly increased ($p < 0.01$) in the AD patients. The CSF sLR11 level in the AD patients was significantly higher ($p < 0.01$) than that of the frontotemporal lobar degeneration patients and the controls. The *APOE-ε4*-positive AD patients have higher sLR11 levels than the *APOE-ε4*-negative patients ($p < 0.01$). ***Conclusions:*** These results suggest that the quantification of CSF sLR11 may serve as a biomarker of AD, although the diagnostic value for individual patients is limited. An elevated CSF sLR11 level in AD patients may be relevant to AD pathogenesis.

Introduction

Lipoprotein receptor LR11 is a member of the LDL receptor family and a type I membrane protein that is highly abundant in the brain. LR11 has a vacuolar protein sorting 10 protein domain that is involved in intracellular protein trafficking. Several lines of evidence suggest that LR11 is intricately implicated in the pathogenesis of Alzheimer disease (AD) [1]. First, variants of the LR11 gene were shown to be a genetic risk factor for sporadic AD [2]. Second, LR11 interacts with the amyloid precursor protein (APP) and alters APP trafficking to discrete compartments such that APP processing to generate amyloid-β (Aβ) is inhibited [3, 4]. Finally, LR11 expression is

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Table 1. Characteristics of patients and control subjects

	AD patients	FTLD patients	Control subjects
Number (male/female)	29 (13/16)	20 (12/8)	27 (12/15)
Age at examination, years	69.4 ± 11.1 (51–85)	69.3 ± 10.5 (52–89)	65.5 ± 12.2 (44–85)
Age at onset, years	64.2 ± 12.2 (47–78)	63.1 ± 10.2 (47–84)	n./a.
MMSE score	15.4 ± 9.3 (0–26)	13.8 ± 8.0 (0–26)	n./a.
CSF tau, pg/ml	520 ± 320 (160–1,150)**	223 ± 77 (118–343)	160 ± 99 (60–325)
CSF Aβ42, pg/ml	84.9 ± 36.1 (26.2–166.6)*	93.2 ± 41.2 (33.3–160.7)	104.2 ± 41.5 (23.0–206.3)
CSF tau/Aβ42	6.6 ± 1.1 (1.6–17.8)**	2.7 ± 1.3 (0.9–5.2)	1.8 ± 1.0 (0.3–3.6)

Values are shown as means ± SD (range). n./a.: data are not applicable.
* $p < 0.05$ compared with control subjects; ** $p < 0.01$ compared with FTLD patients and control subjects by ANOVA, followed by Tukey's post hoc test.

downregulated in the brains of AD patients [5, 6]. These findings prompted us to quantify the soluble LR11 (sLR11) levels in the cerebrospinal fluid (CSF) of patients with AD.

We have recently developed a novel sandwich enzyme-linked immunosorbent assay (ELISA) by producing specific monoclonal antibodies against human LR11, which enabled the sensitive quantification of sLR11 using CSF samples [7]. By this ELISA, we determined the difference in the levels of CSF sLR11 between patients with AD, patients with frontotemporal lobar degeneration (FTLD) and age-matched control subjects.

Materials and Methods

Participants

We studied 29 AD patients, 20 FTLD patients and 27 age-matched control subjects (table 1). The diagnosis of probable AD was made in accordance with the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association [8]. The clinical criteria for the diagnosis of FTLD were based on consensus criteria [9]. Each patient underwent neurological examination, neuroimaging (CT and/or MRI) and laboratory tests to exclude alternative causes of cognitive decline. The *APOE* genotype was determined by *Hha*I digestion of the PCR product in 21 AD patients. CSF samples were also collected from age-matched control subjects, who underwent lumbar puncture to exclude subarachnoid hemorrhage, for lumbar anesthesia or for diagnostic reasons in patients with leukemia in complete remission. The inclusion criteria of the control subjects were absence of memory complaints, preservation of general cognitive functioning and no active neurological and psychiatric diseases. All the samples showed values within the normal ranges including cell count, and glucose, protein and electrolyte levels. Samples were collected after informed consent had been obtained from the participants or their caregivers. The study protocol was approved by the Niigata University School of Medicine.

ELISA of CSF Samples

CSF was collected by lumbar puncture. Samples of CSF were centrifuged at 4,000 *g* for 10 min and the supernatant was immediately frozen in polypropylene tubes and stored at −80°C until use. The storage period of CSF in the freezer ranged from 0 to 3.5 years with an average of 2.1 years. The levels of CSF tau and Aβ42 were determined using commercially available ELISA kits (tau, Innogenetics, Belgium; Aβ42, High-Sensitive, Wako, Japan) as previously reported [10]. The sLR11 level was determined by sandwich ELISA as previously reported [7]. Briefly, a CSF sample (10 μl) diluted with sample buffer was reacted with the capture MAb M3 antibody and then incubated with the biotinylated reporter MAb R14 antibody. The LR11-Mab complex was reacted with horseradish-peroxidase-conjugated streptavidin. A standard curve was constructed using a purified LR11 protein.

Statistical Analysis

All statistical analyses were performed using commercially available software, SPSS 12.0J (SPSS Japan, Japan). The results are shown as means ± standard deviation (SD). Comparisons between groups of subjects were performed by analysis of variance (ANOVA), followed by Tukey's post hoc test. An exploratory correlation was examined using Pearson's correlation coefficient. Receiver operating characteristic curve (ROC) analysis was used to determine the best cutoff values for the measured biomarkers.

Results

There was no statistically significant difference in age at examination or onset among AD patients, FTLD patients and control subjects (table 1). We first quantified tau and Aβ42 levels in CSF, which are well-established CSF biomarkers of AD. As expected, the tau level and tau/Aβ42 of the AD patients were significantly higher than those of the FTLD patients ($p < 0.01$) and control subjects ($p < 0.01$). In addition, the Aβ42 level of the AD patients

Table 2. Diagnostic discrimination between Alzheimer patients and control subjects

CSF biomarker	Cutoff value	Sensitivity, %	Specificity, %	AUC %
sLR11, ng/ml	11.3	66	78	71
sLR11/Aβ42	1.4	71	88	79
Tau, pg/ml	287	82	95	88
Tau/Aβ42	2.4	89	81	85

AUC = Area under the ROC curve.

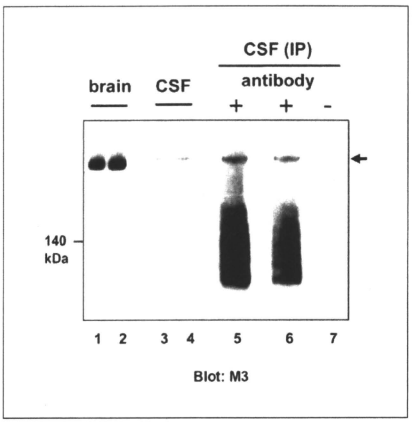

Fig. 1. Characterization of the antibodies against LR11. Human CSF samples (200 μl) from 2 individuals were immunoprecipitated with the R14 antibody (lanes 5 and 6) or without antibody (lane 7). The immune complexes were collected with protein-G-conjugated agarose beads, followed by separating by 6% Tris/glycine polyacrylamide gel electrophoresis. Fractioned proteins transferred to a PVDF membrane were immunoblotted with the M3 monoclonal antibody. Brain samples (lanes 1 and 2) and neat CSFs (10 μl; lanes 3 and 4) were also run on the same gel. A single band migrating at ~250 kDa corresponding to LR11 indicated by an arrow was detected in CSF. An antibody-derived smear band is present in the samples immunoprecipitated with the R14 antibody (lanes 5 and 6) but not in the sample without antibody (lane 7).

was significantly lower than that of the control subjects ($p < 0.05$).

We quantified the CSF sLR11 level of AD patients, FTLD patients and age-matched controls by sandwich ELISA [7]. First, the specificities of the monoclonal anti-LR11 antibodies, M3 and R14, used in ELISA, were examined by Western blot analysis. Immunoblot and immunoprecipitation assays using CSF samples detected a single band migrating at ~250 kDa corresponding to sLR11 (fig. 1). ELISA analysis revealed that the CSF sLR11 level in the AD patients was 13.1 ± 5.6 ng/ml (mean ± SD; median = 12.3; range = 4.6–31.7), which was significantly higher than those in the FTLD patients (8.3 ± 2.4 ng/ml; median = 8.5; range = 3.9–11.5) and controls (9.3 ± 4.5 ng/ml; median = 8.0; range = 3.0–25.9) ($p < 0.01$, fig. 2a). There was no significant correlation of CSF sLR11 level with age at examination or onset, or with Mini-Mental State Examination (MMSE) score. The CSF sLR11 level was comparable among 3 groups of AD patients divided by stage of mild (MMSE = 21–26; sLR11 = 13.2 ± 7.2 ng/ml), moderate (MMSE = 11–20; sLR11 = 12.2 ± 4.3 ng/ml) and severe (MMSE <10; sLR11 = 14.1 ± 3.5 ng/ml) dementia.

We determined the genotype of *APOE* of the AD patients. Sixty-two percent of our AD patients were *APOE*-ε4-positive. *APOE*-ε4-positive AD patients have higher sLR11 levels (13.1 ± 2.9 ng/ml, n = 13, range = 8.6–19.9) than *APOE*-ε4-negative patients (8.1 ± 3.3 ng/ml, n = 8, range = 4.6–15.0) ($p < 0.01$, fig. 2b). Comparable CSF sLR11 levels were found between AD patients heterozygous and homozygous for the *APOE*-ε4 allele.

The sensitivity and specificity of the CSF biomarkers including sLR11, sLR11/Aβ42, tau and tau/Aβ42 were determined by ROC analysis (table 2). ROC analysis of the CSF sLR11 levels in AD and control groups showed a sensitivity of 66%, a specificity of 78% and an area under the ROC curve (AUC) of 71% at a cutoff value of 11 ng/ml. The sLR11/Aβ42 ROC curve analysis increased the sensitivity to 71%, the specificity to 88% and the AUC to 79%. The tau level was the most effective discriminator with a sensitivity of 82%, a specificity of 95% and an AUC of 88% at a cutoff value of 287 pg/ml.

Discussion

Using the sensitive sandwich ELISA that we have recently developed [7], we demonstrated that the CSF sLR11 level is significantly higher in the AD patients than in the FTLD patients and age-matched control subjects. This result suggests that CSF sLR11 may serve as a potential bio-

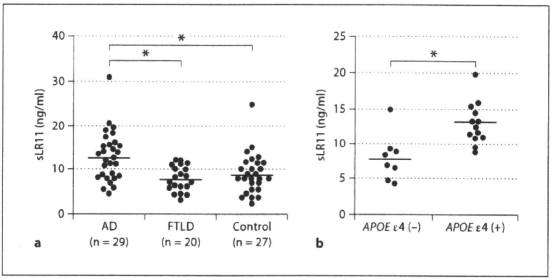

Fig. 2. Quantification of sLR11 in CSF. **a** sLR11 in CSF of AD patients, FTLD patients and age-matched control subjects was quantified by sandwich ELISA. Horizontal lines indicate median values. Statistical differences between groups were calculated by ANOVA, followed by Tukey's post hoc test. * $p < 0.01$. **b** Comparison of CSF sLR11 levels between *APOE*-ε4-positive and *APOE*-ε4-negative patients with AD. Horizontal lines indicate median values. Higher sLR11 levels were observed in *APOE*-ε4-positive than in *APOE*-ε4-negative AD patients (Mann-Whitney U test, * $p < 0.01$).

marker for AD. However, ROC analysis revealed that the measurement of sLR11 in CSF is not as sensitive for diagnostic status of AD as that of total tau; hence, the diagnostic significance of the CSF sLR11 level as a single biomarker might be limited.

In the present study, we showed that *APOE*-ε4-positive AD patients have higher levels of sLR11 than *APOE*-ε4-negative patients. This finding suggests that an elevated CSF sLR11 level in patients may be relevant to AD pathogenesis because the presence of *APOE*-ε4 constitutes a major risk factor for AD. LR11 binds multiple ligands including ApoE and plays a role in the endocytosis of ApoE-containing lipoproteins [11]. In CSF, ApoE is reported to form a complex with Aβ [12]. Interaction between ApoE and LR11 might interfere with the formation of the ApoE-Aβ complex, and this process may enhance Aβ deposition in the brain by increasing the amount of unbound Aβ species. Thus, it will be intriguing to examine the pathogenesis of AD through interaction of LR11 with the action of ApoE4.

In this study, we quantified a soluble form of LR11 in CSF. Full-length membrane-bound LR11 is first processed and activated by a furin-like activity, followed by ligand-induced ectodomain shedding by metalloprotease to release sLR11 [13–15]. A large fraction of the LR11 in the cell membrane is reported to release sLR11 into the culture medium upon proliferation of smooth-muscle cells [14]; however, it is largely unknown how the production of sLR11 is regulated in neuronal cells. In addition, the relationship between the sLR11 level in CSF and membrane-bound LR11 expression in brain parenchyma has not been determined yet. Thus, the mechanism underlying the elevation of CSF sLR11 levels in AD patients warrants further investigation.

In contrast to our results, Ma et al. [16] have recently reported that the CSF sLR11 levels were decreased in AD patients, although a relatively small number of AD patients (n = 19) were examined in their study. The reasons for the discrepancy between our findings and their results of CSF sLR11 level in AD patients have not been completely determined so far; however, a possible explanation may be the difference in the methodology used for the detection of CSF sLR11. In this regard, we took advantage of the availability of the sandwich ELISA using 2 antibodies with different recognition sites to quantify CSF sLR11, whereas Ma et al. [16, 17] performed semi-quantitative analysis by comparing the optical density of bands obtained by immunoblotting of sLR11. An alternative explanation is that Japanese AD patients may have different LR11 expression levels in the brain because an animal study has suggested that the LR11 level in the brain can be increased by a docosahexaenoic-acid-supplemented diet. To address this issue, we examined the LR11 level in the brains of Japanese AD patients (n = 5) in comparison with age-matched control subjects (n = 5). The analysis revealed that the levels of LR11 in the brains of Japanese AD patients were comparable to those in the brains of control subjects (unpublished data), and the results were in contrast to the previous reports [5, 6].

Although our study has potential clinical implications, it also has limitations. First, the diagnosis of the patients was based on clinical criteria, not on autopsy verification. It has been reported that global cognitive function correlated with LR11 expression in the brain, as determined by quantitative immunostaining of LR11 [6], whereas our study showed no correlation between CSF sLR11 level and MMSE score in AD patients. An important question is whether the change in CSF sLR11 levels

is related to LR11 expression levels in the AD brain parenchyma. Second, the difference in sLR11 levels between AD and control groups is relatively small; hence, the diagnostic value for individual patients appears to be limited. Future studies may establish the usefulness of the CSF sLR11 level as a longitudinal biomarker. Lastly, our results were obtained using relatively small sample sets. Clearly, further careful validation studies with larger sample sets will be required for the evaluation of CSF sLR11 as a biomarker of AD.

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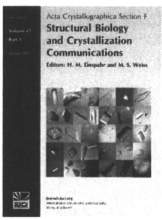

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Crystallization and preliminary crystallographic analysis of human LR11 Vps10p domain

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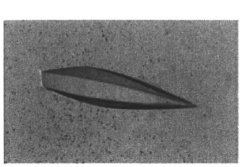

Low-density lipoprotein receptor (LDLR) relative with 11 binding repeats (LR11; also known as sorLA) is genetically associated with late-onset Alzheimer's disease and is thought to be involved in neurodegenerative processes. LR11 contains a vacuolar protein-sorting 10 protein (Vps10p) domain. As this domain has been implicated in protein–protein interaction in other receptors, its structure and function are of great biological interest. Human LR11 Vps10p domain was expressed in mammalian cells and the purified protein was crystallized using the hanging-drop vapour-diffusion method. Enzymatic deglycosylation of the sample was critical to obtaining diffraction-quality crystals. Deglycosylated LR11 Vps10p-domain crystals belonged to the hexagonal space group $P6_122$. A diffraction data set was collected to 2.4 Å resolution and a clear molecular-replacement solution was obtained.

1. Introduction

LDLR relative with 11 binding repeats (LR11) is a type 1 membrane protein that is expressed abundantly in the central as well as the peripheral nervous system and to a lesser extent in other organs (Hermans-Borgmeyer *et al.*, 1998; Yamazaki *et al.*, 1996; Motoi *et al.*, 1999). LR11 expression is selectively reduced in the brains of Alzheimer's disease (AD) patients (Scherzer *et al.*, 2004). Genetic studies have also revealed a strong association of LR11 genetic variants with the risk of AD in several populations (Rogaeva *et al.*, 2007; Lee *et al.*, 2007; Bettens *et al.*, 2008). Although the exact molecular mechanism underlying these phenomena is still unclear, LR11 has been hypothesized to be involved in the intracellular trafficking of amyloid precursor protein (APP) between the trans-Golgi network and early endosomes (Andersen *et al.*, 2005; Willnow *et al.*, 2008), reducing the chance of APP processing in the late endosomes. In fact, overexpression of LR11 in HEK293 cells reduced the levels of extracellular amyloid β peptide produced (Offe *et al.*, 2006). In addition to direct interaction with APP during this trafficking (Andersen *et al.*, 2006), LR11 may also directly interact with β-secretase, blocking the β-secretase–APP interaction (Spoelgen *et al.*, 2006).

Uniquely among the LDLR gene family proteins, LR11 possesses an ~700-amino-acid domain that was initially identified in the vacuolar protein-sorting 10 protein (Vps10p), a sorting protein in yeast that transports carboxypeptidase Y from the Golgi to the vacuole (Marcusson *et al.*, 1994). Mammalian receptors that contain this domain constitute another family of proteins that are highly expressed in neuronal tissues (Willnow *et al.*, 2008). Sortilin is the most extensively studied member of this family and is thought to be involved in the intracellular sorting of brain-derived neurotrophic factor (Chen *et al.*, 2005). The crystal structure of the sortilin Vps10p domain in complex with the neuronal peptide neurotensin has recently been reported (Quistgaard *et al.*, 2009), revealing that the Vps10p domain is comprised of a unique ten-bladed β-propeller fold followed by two small cysteine-rich domains (10CC-a and 10CC-b domains) that make intimate contacts with the bottom face of the propeller. It was also revealed that the ligand peptide is deeply buried in the tunnel of the ten-bladed β-propeller fold. There is no precedent for β-propeller domains with a blade number greater than eight

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

No. of crystals	1
Beamline	Photon Factory BL-17A
Wavelength (Å)	0.980
Detector	ADSC Quantum 270
Crystal-to-detector distance (mm)	279.6
Rotation range per image ( )	0.5
Total rotation range ( )	130
Exposure time per image (s)	5
Resolution range (Å)	50–2.4 (2.44–2.40)
Space group	$P6_122$
Unit-cell parameters (Å)	$a = b = 126.4$, $c = 290.3$
Mosaicity ( )	0.251
Total No. of measured intensities	678636
Unique reflections	54346 (2670)
Multiplicity	12.5 (10.2)
Mean $I/\sigma(I)$	15.9
Completeness (%)	99.6 (100.0)
R_{merge}† (%)	5.5 (46.9)
R_{meas} or $R_{r.i.m.}$ (%)	5.8 (43.8)
Overall *B* factor from Wilson plot (Å^2)	58.3

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th intensity of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations *i* of reflection *hkl*.

and the Vps10p domain is probably the largest of the known extracellular modules that fold as a single structural unit (Bork *et al.*, 1996). Given the potential role of the Vps10p domain in the intracellular trafficking exerted by LR11, it is of great biological as well as medical importance to gain insight into the three-dimensional structure of the LR11 Vps10p domain. To this end, here we report the expression, purification, crystallization and preliminary crystallographic analysis of LR11 Vps10p domain.

2. Methods

2.1. Expression and purification of LR11 Vps10p domain

Human LR11 cDNA (gene accession No. NM_003105) was used to amplify the Vps10p domain, corresponding to the N-terminal 753-residue portion (Ohwaki *et al.*, 2007). The primer sequences were 5′-CCG**GAATTC**GCCACCATGGCGACACGGAGCAG-3′ (the *Eco*RI site is shown in bold) and 5′-CGA**TCTAGA**GGGACAGGGGACCAGCTCTCCTTCC-3′ (the *Xba*I site is shown in bold). The resultant PCR product was cloned into the *Eco*RI/*Xba*I site of pcDNA3.1/Myc-His (Invitrogen) that had been modified to include tag sequences at the C-terminus, SRL*ENLYFQ^GG***HHHHHH-HH**IEQKLISEEDLN**MHTG**HHHHHH**, containing a tobacco etch virus (TEV) protease recognition sequence (shown in italics with the cleavage site indicated by a caret) and tandem His-tag (bold) and Myc-tag (underlined) sequences. Using this plasmid, CHO lec 3.2.8.1 cells (Stanley, 1989) were transfected as described previously (Nogi *et al.*, 2006). The transfected cells were plated in 96-well plates and selected for resistance against 1.5 mg ml^{-1} G418. Confluent culture supernatants from the single-colony wells were subjected to immunoblotting using anti-Myc antibody (Invitrogen) and the clone with the highest secretion level of LR11 Vps10p domain was chosen for production-scale culture in roller bottles. The recombinant protein was initially fractionated from the culture medium by ammonium sulfate precipitation (50% saturation), dissolved in wash buffer (20 m*M* Tris pH 8.0, 0.3 *M* NaCl, 50 m*M* imidazole) and applied onto an Ni–NTA agarose column (Qiagen). The column was washed with wash buffer and eluted with elution buffer (20 m*M* Tris pH 8.0, 0.3 *M* NaCl, 0.25 *M* imidazole). The eluted material was dialyzed in buffer *A* (20 m*M* Tris pH 8.0, 50 m*M* NaCl) and incubated with hexahistidine-tagged TEV protease at an enzyme:substrate ratio of 1:15(*w*:*w*) for 16 h at 277 K to remove the C-terminal tag. For deglycosylation, endoglycosidase H (Endo H; New England Biolabs) was added at a ratio of 5 U enzyme per 1 µg substrate protein and was incubated together with TEV protease under the same conditions as described above. The samples were then reapplied onto the Ni–NTA agarose column to remove the cleaved tags and the protease. The flowthrough fractions were collected and applied onto a Mono Q 5/50 GL column (GE Healthcare). The column was equilibrated with buffer *A* and eluted with a linear gradient of NaCl (50–300 m*M*). LR11 Vps10p domain eluted as a single peak at around 150 m*M* NaCl. The purified protein was concentrated to 6 mg ml^{-1} using an Ultrafree-0.5 centrifugal filter (30 kDa molecular-weight cutoff; Millipore). Typical yields ranged between 0.5 and 1.3 mg per litre of culture supernatant.

2.2. Crystallization

Initial screening for crystallization conditions was carried out using Index, Crystal Screen, Crystal Screen 2 and SaltRx from Hampton Research and Wizard Screens I and II from Emerald BioSystems. In these screens, a Mosquito crystallization robot (TTP Labtech) was used to dispense a mixture of 0.1 µl protein solution (LR11 Vps10p domain in 20 m*M* Tris pH 8.0, 150 m*M* NaCl) and 0.1 µl reservoir solution. Drops were equilibrated against 100 µl reservoir solution using the sitting-drop vapour-diffusion method at 293 K. The initial crystallization condition (solution No. 11 of Crystal Screen; 0.1 *M* sodium citrate tribasic pH 5.6, 1.0 *M* ammonium phosphate monobasic) was further optimized using a 24-well crystallization plate with the hanging-drop vapour-diffusion method. Each well contained 350 µl reservoir solution and the drop consisted of a mixture of 0.5 µl protein solution and 0.5 µl reservoir solution.

2.3. Data collection and phasing

For X-ray diffraction experiments, crystals were soaked in reservoir solution containing 20% glycerol or ethylene glycol and flash-cooled in liquid nitrogen. X-ray diffraction data for the crystal grown from the fully glycosylated sample were collected at 100 K on beamline BL44XU at SPring-8 (Harima, Japan) using a wavelength of 0.900 Å and a DIP-6040 imaging-plate detector (Bruker). An X-ray diffraction data set for the crystal grown from the Endo H-treated protein was collected at 95 K on beamline BL-17A at the Photon Factory (Tsukuba, Japan) using a wavelength of 0.980 Å and an ADSC Quantum 270 CCD detector. For the latter, data collection

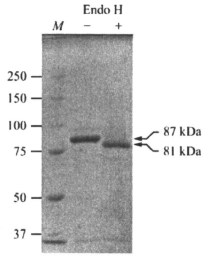

Figure 1
SDS–PAGE analysis of the purified LR11 Vps10p domain before and after Endo H treatment. 2 µg of the purified protein was either treated with 10 U Endo H (+) or left untreated (−) and subjected to SDS–PAGE using 8% gel under reducing conditions followed by staining with Coomassie Brilliant Blue. Lane *M* contains molecular-weight markers (labelled in kDa).

was performed with a total oscillation range of 130° and each diffraction image was obtained with an oscillation angle of 0.5° and an exposure time of 5 s. The diffraction data were processed and scaled using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). Data-collection statistics are listed in Table 1. Phase determination was performed by the molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 2010). The initial model was refined with *REFMAC*5 (Murshudov *et al.*, 1997) and a weighted $2F_{obs} - F_{calc}$ electron-density map was calculated using the coefficients produced by *REFMAC*5. Inspection of the crystal packing and the electron-density map was performed with *Coot* (Emsley & Cowtan, 2004).

3. Results and discussion

LR11 Vps10p domain contains six potential N-linked glycosylation sites and seven disulfide bonds, demanding production in a mammalian-cell expression system. We used CHO lec 3.2.8.1 cells as the production host because they are known to produce glycoproteins with homogeneous glycoforms, which is ideal for crystallization (Davis *et al.*, 1993). The relative molecular mass of the purified protein was estimated to be 87 kDa on an SDS–PAGE gel (Fig. 1). The difference from the calculated molecular weight (76 kDa) is consistent with the presence of glycosylation. Initial crystallization trials were performed using fully glycosylated sample. Crystals were obtained under the condition 0.1 *M* sodium acetate pH 4.5, 1.2 *M* sodium dihydrogen phosphate at 293 K and grew to dimensions of approximately 30 × 30 × 100 µm within two months (Fig. 2*a*). Processing of the X-ray diffraction images indicated that the unit-cell parameters of the crystal were $a = b = 125.8$, $c = 292.7$ Å (Fig. 2*a*). However, the resolution limit of X-ray diffraction was very low (below 6 Å) and we could not determine the atomic resolution structure using these crystals. We reasoned that the presence of highly flexible glycan chains in the crystal prevented ordered tight packing of the LR11 Vps10p domain. Therefore, we trimmed the glycan

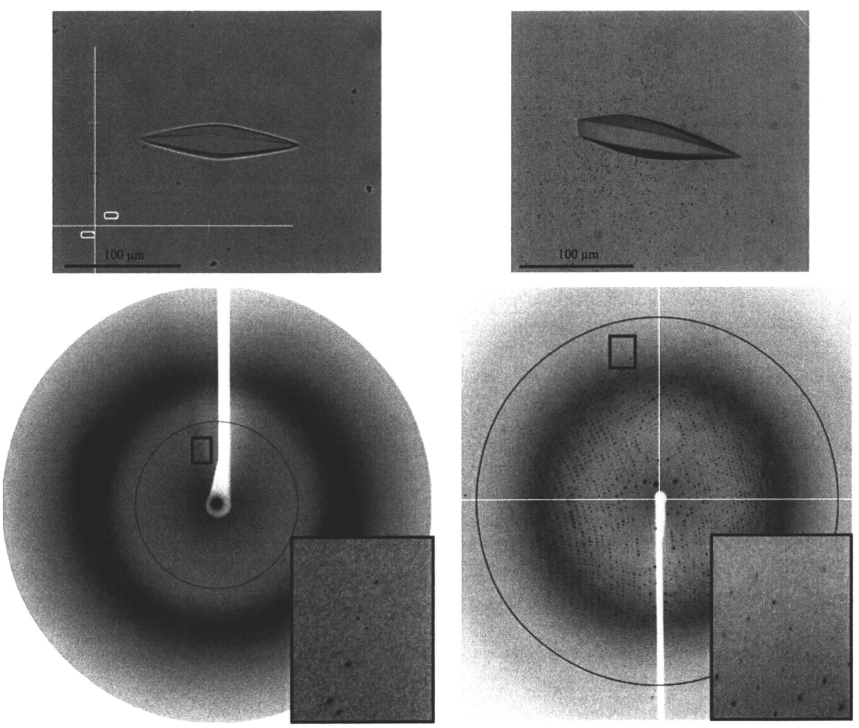

Figure 2
Crystals and diffraction patterns obtained using samples before (*a*) and after (*b*) Endo H treatment. 6.0 Å (*a*) and 2.4 Å (*b*) resolution circles are shown. Expanded views of the regions marked with a rectangle are shown in the insets.

chains from the purified protein using Endo H. Endo H cleaves between the two GlcNAc residues at the base of N-glycans, leaving a single GlcNAc residue at each glycosylation sequon that maintains overall protein solubility. Although Endo H exhibits varying degrees of cleavage efficiency towards different glycan chains, it is able to efficiently cleave the $Man_5GlcNAc_2$ chain, which is the predominant glycoform present on proteins expressed in CHO lec 3.2.8.1 cells (Davis *et al.*, 1993). Endo H treatment of LR11 Vps10p domain resulted in a reduction of the relative molecular mass by ~6 kDa, which is consistent with the complete removal of six glycan chains (Fig. 1). The deglycosylated sample could be crystallized under the same conditions and with the same incubation period (~2 months) as the untreated sample (Fig. 2*b*). Remarkably, the quality of the crystals was dramatically improved and the crystals diffracted to 2.4 Å resolution (Fig. 2*b*), while the unit-cell parameters ($a = b = 126.4$, $c = 290.3$ Å) remained essentially unchanged from the original crystal of fully glycosylated sample (Table 1).

The symmetry and systematic absences of the diffraction intensities indicated that the new crystal of deglycosylated LR11 Vps10p domain belonged to either space group $P6_122$ or its enantiomer $P6_522$. In an attempt to determine the initial phases, the molecular-replacement method was performed using the atomic coordinates of the sortilin Vps10p domain (PDB code 3f6k; Quistgaard *et al.*, 2009) as a search model. Specifically, the β-propeller module was extracted from the coordinates. In addition, residues and atoms that do not map onto the sequence of the LR11 Vps10p domain were deleted from the model with the 'FILE_SEQUENCE' option of *MOLREP*. While the sortilin Vps10p domain shows only 25% sequence identity to the LR11 Vps10p domain, it gave a clear solution in $P6_122$ for which the peak-to-noise ratios of the rotation and translation functions were 5.46 and 24.85, respectively. One monomer was located in the asymmetric unit, showing an R factor of 62.8%. The resulting model was subjected to initial rigid-body and subsequent restrained refinement, which resulted in a reduction of the R and R_{free} factors to 55.5% and 55.9%, respectively. Preliminary model building revealed that all of the potential N-linked glycosylation sites are located on the surface of the protein and we could identify clear electron density that is likely to represent the remaining GlcNAc moiety at five of the six sites. Furthermore, three of these sites are very close to the neighbouring molecules. Although the three N-glycans are not directly involved in crystal packing, it is possible that deglycosylation with Endo H stabilized the packing by lowering the disorder arising from the flexibility of the carbohydrates. In fact, the Matthews coefficient (Matthews, 1968) and solvent content (4.4 $Å^3\ Da^{-1}$ and 72.2%, respectively) suggested a loose crystal packing (Kantardjieff & Rupp, 2003), indicating the presence of a large space that can accommodate flexible N-glycans. Further model building and structure refinement are now in progress and the mechanism by which the deglycosylation improved the crystal quality may be clarified after completion of the model assignment, including the carbohydrates.

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