Table 2 Clinical features of 20 male and 18 female patients with Danon disease

Characteristics	Male	Female
Subjects, n	20	18
Age, y, mean ± SD	17 ± 7 (n = 20)	38 ± 12 (n = 14)
Age at onset, n		
Infantile	4	
Childhood	11	
Second decade	5	
Age at death, y, mean ± SD	19 ± 6 (n = 7)	40 ± 7 (n = 6)
Cause of death, n (%)		
Cardiac failure	7/7 (100)	6/6 (100)
Myopathy, n (%)	18/20 (90)	6/18 (33)
Muscle weakness	16/20 (80)	6/18 (33)
Fatigability only	2/20 (10)	0/18 (0)
Cardiomyopathy, n (%)	20/20 (100)	18/18 (100)
Hypertrophic	16/19 (84)	2/7 (29)
Dilated	2/19 (11)	5/7 (71)
Mixed	1/19 (5)	
Mental retardation, n (%)	14/20 (70)	1/18 (6)
Elevated CK, n (%)	18/18 (100)	5/8 (63)
Serum CK (IU/L), mean ± SD	1574 ± 790 (n = 18)	
Serum AST (IU/L), mean ± SD	382 ± 234 (n = 12)	
Serum ALT (IU/L), mean ± SD	344 ± 199 (n = 11)	
Serum LDH (IU/L), mean ± SD	1874 ± 935 (n = 11)	
Serum aldolase (IU/L), mean ± SD	17.4 ± 8.0 (n = 9)	
Abnormal EKG, n (%)	17/17 (100)	10/10 (100)
Age at abnormal EKG, y, mean ± SD	13 ± 5 (n = 10)	
Abnormal echocardiogram, n (%)	19/19 (100)	3/3 (100)
Pacemaker, n (%)	6/20 (30)	3/18 (17)
Heart transplantation, n (%)	1/20 (5)	2/18 (11)
Myogenic EMG, n (%)	10/10 (100)	
Hepatomegaly, n (%)	5/14 (36)	
Splenomegaly, n (%)	1/13 (8)	
Foot deformity, n (%)	3/8 (38)	
Delayed milestone, n (%)	3/8 (38)	
Abnormal EEG, n (%)	2/7 (29)	
Manifesting mother, n (%)	14/20 (70)	6/18 (33)

CK = creatine kinase; AST = aspartate transaminase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; EMG = electromyography.

gression of the disease. For example, two male patients were first identified when isolated increases in serum creatine kinase were noted, and two others because they had abnormal EKG findings preceding cardiac symptoms. Only one male patient developed dyspnea in the infantile period; most commonly onset is of childhood onset. All patients were born through normal pregnancy and delivery. Delayed milestones with mild mental retardation were observed in three male patients. Five male patients ran slower than their peers during childhood. The mean age at death was 19 years, with a range of 12 to 29 years; all died of cardiac failure or sudden cardiac arrest. No male patients survived beyond age 29 years except for a 34-year-old man in Family 1. He had mild cardiomyopathy but reported no cardiac symptoms.

All 20 male patients had cardiomyopathy. Most had hypertrophic cardiomyopathy, but two had dilated cardiomyopathy, and one had mixed features of hypertrophic and dilated cardiomyopathy. Permanent pacemakers were inserted in six male patients (30%). Heart transplantation was performed in one male patient (5%).

Myopathy was usually mild and was observed in 17 of 20 male patients (85%). The main symptoms were mild proximal limb and neck muscle weakness. All male patients remained ambulatory. Two male patients without weakness had only premature fatigability without fixed weakness. When present, muscle atrophy was mild. Only three male patients had obvious muscle wasting.

Mild mental retardation was observed in 14 male patients (70%). IQ was reduced to a range of 60 to 91 in five male patients in whom formal cognitive tests were performed.

On physical examination, hepatomegaly was observed in five of 14 male patients (36%) and splenomegaly in one of 13 male patients (8%). Foot deformities, such as pes cavus, was seen in three of eight male patients (38%).

Laboratory tests showed that serum creatine kinase and aldolase were increased five- to 10-fold above upper limits of normal. Creative kinase values ranged from 339 to 3,128, with an average of 1,574 ± 790 IU/L (normal range: <220). In addition, serum aspartate transaminase, alanine aminotransferase, and lactate dehydrogenase levels were also increased (see table 2). On biochemical analyses, acid α-1,4-glucosidase activity was normal in most male patients but was increased in some.

EKG findings were abnormal in all male patients tested (n = 17, 100%). The most frequent pathologic finding was Wolff-Parkinson-White (WPW) syndrome (six male patients) with a short PQ interval, a prolonged QRS complex, and a delta wave. Male patients with hypertrophic cardiomyopathy showed abnormally high voltage in precordial leads. In addition, giant negative T wave, third-degree atrioventricular block, atrial flutter, bradycardia, abnormal Q wave, and complete left bundle branch block were also observed. The mean age at which abnormal EKG findings were first noticed was 13 ± 5 years (n = 10). EKG also showed abnormalities in all male patients studied (n = 19); most had concentric hypertrophic cardiomyopathy with impaired left ventricular function. Seven male patients had abnormally thick interventricular septum and posterior walls. Electromyography showed small-amplitude short-duration motor unit potentials in all 10 male patients tested. In addition, fibrillation and positive sharp waves were present in three male patients, myotonic potentials at rest were noted in three individuals, and all three forms of abnormal discharges were noted in one. Nerve conduction studies were normal in all five male patients tested. Electroencephalography showed mild abnormalities in two of seven male patients; one had moderate bitemporal slowing and the other had slow α wave pattern with emergence of diffuse θ waves during sleep.

Table 3 Immunohistochemical and histochemical characteristics of skeletal muscle in Danon disease

Characteristic	Expression	Location
NSE	+	Vacuolar membrane
AchE	+	Vacuolar membrane
Acid phosphatase	+	Vacuolar material
Dystrophin	+	Vacuolar membrane and sarcolemma
Laminin	+	Vacuolar membrane and sarcolemma
Sarcoglycan	+	Vacuolar membrane and sarcolemma
LAMP-2	−	

NSE = nonspecific esterase; AchE = acetylcholinesterase.

Fourteen of the 20 mothers were symptomatic (70%). Four of the five asymptomatic mothers (80%) had single affected sons. *LAMP-2* gene sequences were normal in blood DNA of two asymptomatic mothers. The clinical features of 18 affected women are summarized in table 2.

It is difficult to define the age at onset in affected women because of the insidious nature of the disease. In the present study, the age at onset in the affected women was 38 ± 12 years (range, 12 to 53 years). Six women died (average age of 40 ± 7 years) due to cardiac failure or cardiac arrest.

Cardiomyopathy was evident in all 18 affected women. Two had hypertrophic cardiomyopathy and five had dilated cardiomyopathy. Permanent pacemakers were placed in three affected women; two subsequently underwent heart transplantation. Myopathy was observed in six women (33%) with mild proximal weakness in proximal limb or neck muscles. Mild mental retardation was noted in one female patient (6%).

Serum creatine kinase was increased in five female patients and ranged from 76 to 643 IU/L. EKG findings were abnormal in all female patients; findings included left ventriclular hypertrophy, first-degree atrioventricular block, sick sinus syndrome, atrial flutter, skipped beat, abnormal Q wave, and complete left bundle branch block.

Family 1 has a frame-shift mutation in exon 9b of the *LAMP-2* gene, which is predicted to affect only the LAMP-2b isoform whereas all other mutations affect both LAMP-2a and 2b isoforms. The two affected members in that family have hypertrophic cardiomyopathy and mild myopathy but no mental retardation. Both female patients are currently alive, ages 23 and 34 years. Two heterozygous women are 56 and 27 years of age and have not developed cardiac abnormalities, muscle weakness, or elevations of serum creatine kinase.

Muscle pathology. Pathologic findings in muscle are summarized in table 3. All probands showed mild to moderate variation in fiber size. Small vacuoles were seen in many fibers, which may appear as basophilic granules rather than vacuoles in hematoxylin and eosin preparations (figure 1). The granules contained acid phosphatase–positive material. In all probands, acetylcholine and nonspecific esterase activities were associated with the vacuolar membranes. Furthermore, on immunohistochemistry the vacuolar membranes stained with antibody against dystrophin, whereas LAMP-2 was completely absent in muscle from all male patients (figure 2).

On electron microscopy, pathologic findings in muscle included numerous intracytoplasmic autophagic vacuoles with glycogen particles and cytoplasmic debris. In addition, some of the vacuoles were bounded by membranes with features of the basal lamina.

Discussion. We identified 13 families, with a total of 20 male and 18 female patients, with genetically confirmed Danon disease. All 13 probands were male, whereas women were less severely affected, with later-onset cardiomyopathy. These findings suggest that Danon disease is an X-linked dominant disorder. In fact, the causative gene for Danon disease, *LAMP-2*, has been mapped to chromosome

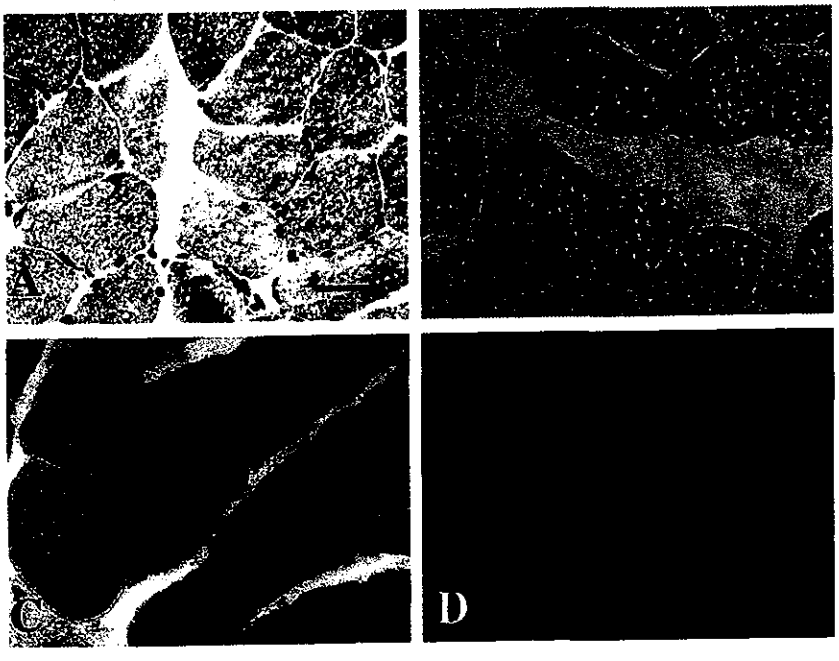

Figure 1. Histochemistry and immunohistochemistry. Transverse sections of skeletal muscle biopsy specimens from affected men with Danon disease. Several fibers with tiny basophilic intracytoplasmic vacuoles are scattered throughout (A). The vacuolar membrane has high acetylcholinesterase (B) and nonspecific esterase (C) activities. Dystrophin expressed in vacuolar membrane and sarcolemma of muscle fibers (D). (A) Hematoxylin and eosin stain. (B) Acetylcholinesterase. (C) Nonspecific esterase. (D) Immunohistochemistry with antibody against dystrophin. Bar = 40 μm.

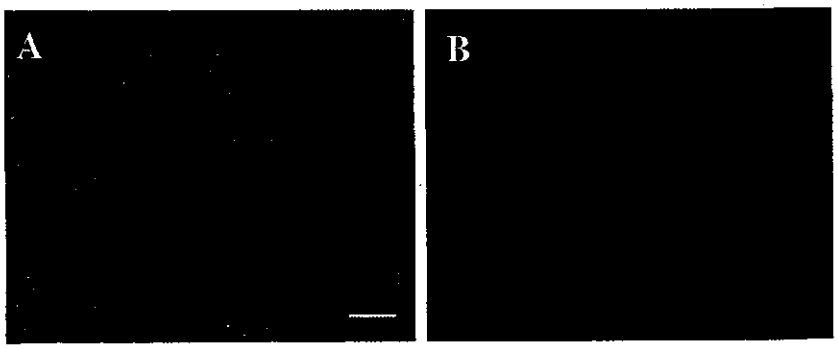

Figure 2. Immunohistochemistry. Transverse sections of skeletal muscle biopsies from control subjects (A) and affected men from Danon disease (B) were immunostained with antibody against LAMP-2. LAMP-2 is present in the control muscles, whereas it is absent in Danon disease muscles. Bar = 40 μm.

Xq24.[3] In previous studies, this disease had been defined clinically by the triad of cardiomyopathy, myopathy, and variable mental retardation. We have confirmed that this clinical triad encompasses the main clinical features of Danon disease.

Myopathy is usually mild and can be clinically silent; however, all male patients had elevated serum creatine kinase levels and myogenic changes by electromyography. Symptomatic patients typically had proximal limb weakness, which was very slowly progressive or stable. Myopathic symptoms were noted in only a few female patients and were even milder. Two female patients without overt myopathy underwent muscle biopsy; one showed no remarkable findings, but the other revealed focal vacuolation in muscle fibers.[5,11]

Electrophysiologically, in addition to myopathic units in all male patients tested, myotonic discharges were observed in three of 10 male patients; however, clinical myotonia was not evident in any patient. Therefore, myotonic discharges are not uncommon in patients with Danon disease but are observed less frequently than in patients with type II glycogenesis.[17]

Cardiac symptoms are the dominant clinical features and the most important prognostic factors, because all of the deceased patients died of cardiac failure. Histologically, cardiomyocytes have shown severe vacuolation and degeneration, including myofibrillar disruption and lipofuscin accumulation.[5,11]

Most male patients developed hypertrophic cardiomyopathy, whereas most female patients showed dilated cardiomyopathy. Previous reports described the evolution of the hypertrophic into dilated cardiomyopathy with progressive cardiac failure.[1,7] Therefore, dilated cardiomyopathy may be associated with the timing of cardiac investigation. However, other factors may determine the type of cardiomyopathy.

The WPW EKG pattern observed in Danon disease has been attributed to myocardial hypertrophy rather than to the presence of an accessory pathway.[6] However, the incidence of WPW is relatively high (6/17 male patients, 35%) in Danon disease as compared with that in other hypertrophic cardiomyopathy, such as idiopathic hypertrophic cardiomyopathy (1.5%) and familial hypertrophic cardiomyopathy (12%),[18] which suggests the presence of a specific pathomechanism for preexcitation in Danon disease rather than a simple association with cardiac hypertrophy.

Although permanent pacemakers were inserted in several patients, heart transplantation may be the most effective intervention. The only male patient who underwent heart transplantation was a 25-year-old man with hypertrophic cardiomyopathy, atrial flutter, and atrioventricular block. For 4 years after the operation, he did not have significant deteriorations except occasional rejection episodes.[11] As in men, cardiomyopathy can be fatal in female patients. This suggests that not only male patients but also women with Danon disease should be considered for heart transplantation. In addition, we suggest that asymptomatic female relatives of male patients should be investigated for cardiomyopathy and followed closely to detect early signs of a potentially life-threatening condition.

Mild mental retardation was observed in more than half of the male patients and electroencephalography showed mild abnormalities in two male patients. One male patient had decreased cerebral glucose metabolism in the cerebral cortex on PET.[13] There were no CNS manifestations and no patient showed structural brain abnormalities by CT or MRI. However, careful postmortem neuropathologic analysis is needed to further characterize the CNS involvement in Danon disease.

Muscle pathology is characterized by small basophilic vacuoles in many fibers. The vacuolar membranes show acetylcholine and nonspecific esterase activities that are very useful to distinguish Danon disease from other vacuolar myopathies including acid maltase deficiency. The positive acid phosphatase stain is also a useful though not specific diagnostic feature. However, similar vacuoles are also seen in other autophagic vacuolar myopathies including X-linked myopathy with excessive autophagy[19] and infantile autophagic vacuolar myopathy.[8] The pathognomonic finding in Danon disease is the absence of LAMP-2 immunohistochemical staining.

In *LAMP-2* knockout mice, a wider variety of organs is affected, including liver, kidney, pancreas, small intestine, thymus, and spleen, in addition to heart and skeletal muscle.[20] Therefore, other organs may also be involved in Danon disease. In fact, some male patients had hepatomegaly and had dispropor-

tionately high serum aspartate transaminase and alanine aminotransferase levels compared with creatine kinase values. Mild portal fibrosis with normal hepatocytes was seen in liver from one male patient with cardiac dysfunction.[11] However, another biopsy specimen from another male patient without cardiopathy showed sclerotic portal and central veins, nuclear vacuolization of the hepatocytes, and enlarged mitochondria with irregular cristae,[12] suggesting that liver may be primarily affected. In addition, there was platelet dysfunction and glycogen accumulation in one other patient.[9]

Human exon 9 of the *LAMP-2* gene exists in two forms, exon 9a and 9b, that are alternatively spliced and produce two isoforms, LAMP-2a and LAMP-2b. LAMP-2a is present ubiquitously, whereas LAMP-2b is expressed predominantly in heart and skeletal muscles.[15] The mutation in Family 1 affects only the LAMP-2b isoform, whereas all other mutations affect both LAMP-2a and 2b isoforms. The two affected men in Family 1 had hypertrophic cardiomyopathy and mild myopathy but no mental retardation. In this family, one affected man is alive at age 34 years and one female patient is alive at age 56 years, suggesting that isolated deficiency of the LAMP-2b isoform may be associated with a milder phenotype.

Most mothers of the probands have shown cardiac abnormalities. This finding is in sharp contrast to the broad range of clinical severity in manifesting carriers of other X-linked recessive disorders, such as Duchenne muscular dystrophy. Although we identified five asymptomatic mothers, all but one (80%) had sporadic affected sons, raising the possibility that these singleton male patients were due to spontaneous mutations. In support of this notion, two of the asymptomatic mothers did not harbor a mutation in blood DNA. All available evidence indicates that Danon disease is an X-linked dominant disease.

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Maturation of the activities of recombinant mite allergens Der p 1 and Der f 1, and its implication in the blockade of proteolytic activity

Toshiro Takai[a,*], Reiko Mineki[b], Takuya Nakazawa[a], Masatoshi Takaoka[c], Hiroshi Yasueda[d], Kimie Murayama[b], Ko Okumura[a,e], Hideoki Ogawa[a,f]

[a] *Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[b] *Division of Biochemical Analysis, Central Laboratory of Medical Sciences, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[c] *Saitama Institute of Public Health, 639-1, Kamiokubo, Saitama 338-0824, Japan*
[d] *Clinical Research Center, National Sagamihara Hospital, 18-1 Sakuradai, Sagamihara, Kanagawa 228-8522, Japan*
[e] *Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*
[f] *Department of Dermatology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan*

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Abstract Recombinant pro-Der p 1 expressed in yeast *Pichia pastoris* was convertible into the prosequence-removed mature Der p 1 with full activities of cysteine protease and IgE-binding with or without N-glycosylation of the mature sequence as well as pro-Der f 1. The active recombinant variants will be the basis for various future studies. The major N-terminus of pro-Der p 1 with low proteolytic activity was the putative signal-cleavage site, while that of pro-Der f 1 contained not only the equivalent site but also 21 residues downstream, and pro-Der f 1 retained significant activity. Contribution of the N-terminal region of the Der p 1 prosequence including an N-glycosylation motif on effective inhibition of proteolytic activity of pro-Der p 1 was suggested.

Key words: Recombinant major house dust mite group 1 allergen; Maturation; Prosequence; *N*-Glycosylation; Cysteine protease; IgE-binding

1. Introduction

House dust mites of the *Dermatophagoides* species (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are the most important causative factor associated with various allergic diseases among all the indoor allergens [1–3]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens are considered the major house dust mite allergens [4–7]. IgE-binding activity for both group 1 and group 2 allergens is detected in the majority of sera from patients allergic to house dust mites. Group 1 allergens, which belong to the papain-like cysteine protease family, are mainly found in mite feces and are thought to function as digestive enzymes in the intestine of mites [8–10]. IgE-binding to the group 1 allergens is highly dependent on the tertiary structure of the allergens [5,11,12]. Recently, the cysteine protease activity of a group 1 allergen from *D. pteronyssinus*, Der p 1, has been proposed to be involved in the pathogenesis of allergy [13–17]. Therefore, enzymatically and immunologically active recombinant group 1 allergens with structures equivalent to the natural ones will be useful tools for diagnosis, standardized allergen-specific immunotherapy (AIT) [18–20], and various studies, including the analysis of conformation-dependent IgE epitopes [21–27], determination of tertiary structure [28], allergen engineering for safer and more effective AIT [18–20,23–26,29], resolving the relationship between the enzymatic activity and pathogenesis [13–17,30], and so on.

The cDNAs for the mite group 1 allergens and their isoforms have been cloned [31–34]. They encode the preprosequence at the N-terminus of the mature sequence. Recently, we have developed efficient systems to prepare recombinant forms of the group 1 allergen from *D. farinae*, Der f 1, which is expressed abundantly in methylotrophic yeast *Pichia pastoris* [35,36] and bacteria [37], and demonstrated their mature protease activity, IgE reactivity, and an apparent molecular weight equivalency to that of natural Der f 1. On the other hand, preparation of recombinant mature Der p 1 with equivalent properties in terms of activities and molecular weight has not been reported, although enzymatically inactive pro-Der p 1 was reported by some researchers [38–40], and highly glycosylated mature Der p 1 with enzymatic and allergenic activities but with a broad apparent molecular weight much higher than natural Der p 1 was reported recently [41].

We show that recombinant proforms of Der p 1 as well as those of Der f 1 expressed in yeast *P. pastoris* with or without N-glycosylation of the mature sequence are convertible into the prosequence-removed mature forms with full cysteine protease activity. IgE reactivities of the recombinant mature forms were also equivalent to those of natural ones. This is the first report that demonstrates the preparation of recombinant mature Der p 1, which has no yeast-derived N-glycosylation and retains an apparent molecular weight equivalency to natural Der p 1, with full enzymatic and IgE-binding activities. Moreover, we compare the properties of the recombinant pro- and mature forms of the two homologous allergens Der p 1 and Der f 1 and discuss the role of the N-terminal region of the prosequence in the maturation and inhibition of proteolytic activity of the recombinant proforms of the house dust mite group 1 allergens.

*Corresponding author. Fax: (81)-3-3813 5512.
E-mail address: t-takai@med.juntendo.ac.jp (T. Takai).

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2. Materials and methods

2.1. Cloning of cDNA coding for prepro-Der p 1 and prepro-Der f 1

The total RNA from cultured mites was prepared, and the cDNA fragments of prepro-Der p 1 and prepro-Der f 1 were synthesized and amplified by reverse transcription- polymerase chain reaction (RT-PCR). The PCR primers were designed on the basis of previously described cDNA sequences [33,34]. The cDNA fragments were subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA, USA) or pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands) and sequenced by a DNA sequencer (ABI Prism 377, Applied Biosystems, Foster City, CA, USA).

2.2. Construction of expression vectors encoding prepro-Der p 1, prepro-Der f 1, and their mutants

A *Bam*HI site and a *Not*I site were added to the 5′ and 3′ termini, respectively, of each open reading frame (ORF) for prepro-Der p 1 and prepro-Der f 1 by PCR. Vectors for expression in the methylotrophic yeast *P. pastoris* encoding the prepro-Der p 1-WT and prepro-Der f 1-WT, each of which has the intact wild-type sequence, were constructed by insertion of the PCR-amplified cDNA fragments digested with *Bam*HI and *Not*I to the *Bam*HI/*Not*I site of pPIC3.5 (Invitrogen). Expression vectors encoding for mutants whose N-glycosylation motifs within the mature sequences are disrupted, prepro-Der p 1-N52Q and prepro-Der f 1-N53Q, were constructed by site-directed mutagenesis. It was confirmed that the inserted sequences encoded the correct amino acid sequences by DNA sequencing.

2.3. Expression of prepro-Der p 1, prepro-Der f 1, and their mutants in P. pastoris

The method was as described previously [35] with minor modifications. Briefly, the expression vectors were introduced into *P. pastoris* GS115 cells by electroporation. Transfectants in which the locus of the genomic alcohol oxidase gene was homologously recombined with the linearized vector DNA were selected. The transfectants were cultured at 30°C in 5 ml of YPD. Four milliliters of the one or two overnight full-growth culture was inoculated in 400 ml of BMGY and cultured for 24 h. The cells were pelleted by centrifugation, resuspended in 80 ml of BMMY and cultured for an additional 72–96 h with addition of methanol to the culture every 24 h to maintain the continuous expression of the recombinant products. The culture supernatant was harvested by centrifugation. The culture supernatants, to which a 1/5-volume of 0.5 M Tris–HCL (pH 9.0) containing 0.1% NaN_3 was added, were concentrated by ammonium sulfate-precipitation. The concentrations of ammonium sulfate were 80%-saturation for pro-Der p 1-WT and pro-Der f 1-WT, and 50%-saturation for pro-Der p 1-N52Q and pro-Der f 1-53Q. The precipitates were dissolved in 50 mM Tris–HCL (pH 9.0) containing 0.01% NaN_3 at 1/20 volume of the original culture supernatants.

2.4. Maturation of pro-Der p 1, pro-Der f 1, and their mutants, and purification of the pro- and mature forms

The method was as described previously [35] with minor modifications. Briefly, the supernatants concentrated by ammonium sulfate-precipitation were dialyzed against 100 mM sodium acetate buffer (pH 4.0) for 3 h at room temperature and an additional 24–48 h at 4°C. After this activation process, the recombinant products were subjected to anion exchange column chromatography after dialysis against a buffer for the chromatography. Purities of the fractions of NaCl-gradient elution (0–400 mm) were checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The fractions containing pure recombinant proteins were mixed, aliquoted, and stored at −80°C. Protein concentrations were determined by the Bradford's procedure using a protein assay kit (Bio-Rad, Richmond, CA, USA) with bovine IgG (Bio-Rad) as the standard. The recombinant proforms of Der p 1 and Der f 1 were purified by the same methods as the mature forms but without the activation process of dialysis against the acidic buffer.

2.5. Protein sequencing

The method was as described previously [42,43]. The samples were subjected to SDS–PAGE and electroblotted onto membranes. Before the SDS–PAGE, the samples were boiled under reducing conditions with dithioerythritol in the sample buffer, and then the cysteine residues were alkylated [43]. The membranes were stained with Coomassie Brilliant Blue (CBB). The visualized bands were subjected to N-terminal amino acid sequencing using protein sequencers G1005A (Hewlett Packard, Palo Alto, CA, USA) and/or Procise 492cLC (Applied Biosystems).

2.6. Purification of natural Der p 1 and Der f 1

Natural Der p 1 and Der f 1 were purified from the whole culture extracts from house dust mites, *D. pteronyssinus* and *D. farinae*, respectively, as described previously by Yasueda et al. [5,6]

2.7. Measurement of proteolytic activity

Cysteine protease activity was measured as described previously [44] with modifications. Assays were conducted in reaction buffer (50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA) with or without 1 mM dithiothreitol (DTT) at 37°C. Final concentrations of the recombinant or natural allergens were 100 nM. The substrate butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-Gln-Ala-Arg-MCA) (Peptide Institute, Osaka, Japan) was used at 0.1 mM. The fluorescence of aminomethylcoumarin released from the substrate was measured at an excitation of 355 nm and emission of 460 nm on a fluorometer, Fluoroskan Ascent (Labsystems, Vantaa, Finland).

2.8. Radioallergosorbent test-enzyme immunoassay (RAST-EIA)

IgE reactivities were measured by radioallergosorbent test-enzyme immunoassay (RAST-EIA) using a RAST-EIA Kit (Pharmacia, Peapack, NJ, USA) as previously described [24]. Briefly, serum of each allergic patient (1/8 dilution) was incubated with paper discs coupled with the allergens. Then, the IgE that bound to the disc was detected with β-galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence at an excitation of 355 nm and emission of 460 nm on the fluorometer. The mite-allergic patients' sera were kindly provided by Dr. Hirokazu Okudaira, Tokyo University.

3. Results

3.1. Cloning of cDNAs encoding for prepro-Der p 1 and prepro-Der f 1

The cDNAs encoding for prepro-Der p 1 and prepro-Der f 1 were cloned by RT-PCR. Polymorphism was found in the nucleotide sequences of the ORF of the clones. The amino acid sequence of the prepro-Der p 1 clone selected for expression in this study was identical with that previously reported as 'clone (c)' by Chua et al. [33] except for one residue (−95 in the signal sequence). The amino acid residue at position −95 was threonine in this isoform of prepro-Der p 1, whereas it was valine in 'clone (c)' reported by Chua et al. The amino acid sequence of the prepro-Der f 1 clone selected for expression in this study was identical with that previously reported by Yasuhara et al. [34].

3.2. Preparation of purified recombinant and natural types of Der p 1 and Der f 1

The prepro-Der p 1 and prepro-Der f 1 with original mite amino acid sequences (Fig. 1A), prepro-Der p 1-WT and prepro-Der f 1-WT, and their mutants whose N-glycosylation motif within the mature sequences was disrupted by the substitution of an asparagine residue in the motif to a glutamine residue, prepro-Der p 1-N52Q and prepro-Der f 1-N53Q, were expressed. The proforms were secreted into culture medium, and processed to mature forms by the methods described in Materials and methods. Processing of the proforms was checked by SDS–PAGE before the anion exchange chromatography. Essentially, all the allergen was processed during dialysis against the acidic buffer. The amounts of purified recombinant products per 1 l of BMMY medium were 80–400 mg of pro-Der p 1-WT and pro-Der f 1-WT, 60–210 mg of mature Der p 1-WT and Der f 1-WT, 15–70 mg of pro-Der p 1-N52Q and pro-Der f 1-N53Q, and 10–40 mg of mature Der p 1-N52Q and Der f 1-N53Q.

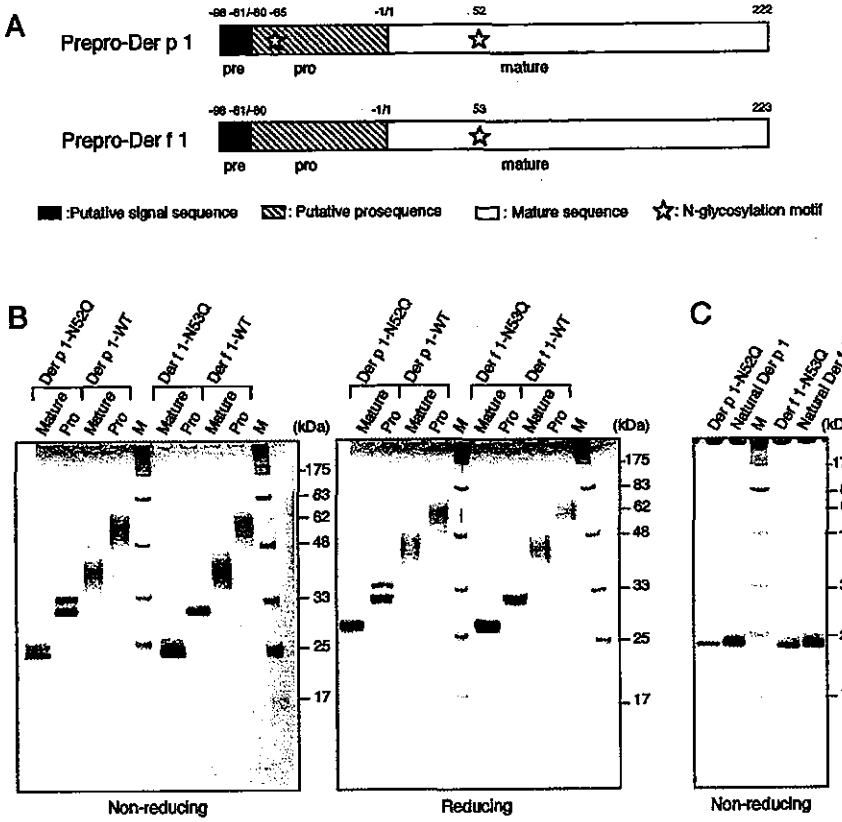


Fig. 1. Recombinant and natural types of the major house dust mite group 1 allergens Der p 1 and Der f 1. A: Schematic representation of prepro-Der p 1 and prepro-Der f 1 expressed in this study, which have the original mite amino acid sequences. B: Purified recombinant forms of Der p 1 and Der f 1. Proforms were purified from concentrated supernatants of yeast *P. pastoris* by column chromatography. Mature forms were prepared from the proforms in concentrated supernatants by dialysis against an acidic buffer and purified by column chromatography. They were subjected to SDS–PAGE (15–25%) and stained with CBB. Non-reducing and reducing: samples were boiled in the absence and presence of 2-mercaptoethanol in the sample buffer, respectively. C: Comparison of recombinant mature forms without N-glycosylation, Der p 1-N52Q and Der f 1-N53Q, and natural types of Der p 1 and Der f 1. Natural types of Der p 1 and Der f 1 were purified from mites. Mature: mature form; pro: proform; M: molecular weight marker; N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence.

The results of SDS–PAGE of purified recombinant forms of Der p 1 and Der f 1 are shown in Fig. 1B. Two bands were observed in the lane for pro-Der p 1-N52Q. The bands for recombinant proteins with an N-glycosylation motif in the mature sequence showed as smears (Der p 1-WT, pro-Der p1-WT, Der f 1-WT, and pro-Der f 1-WT). Mobilities of the mature forms under reducing conditions were significantly less than those under non-reducing conditions. Mobilities of the proforms under reducing conditions were equivalent to or slightly less than those under non-reducing conditions. Approximate apparent molecular weights based on the mobilities of the bands on the SDS–PAGE under non-reducing conditions were 24 kDa for mature Der p 1-N52Q and Der f 1-N53Q, 31 kDa and 33 kDa for pro-Der p 1-N52Q, 31 kDa for pro-Der f 1-N53Q, 35–45 kDa for mature Der p 1-WT and Der f 1-WT, and 50–70 kDa for pro-Der p 1-WT and pro-Der f 1-WT. Those under reducing conditions were 27 kDa for mature Der p 1-N52Q and Der f 1-N53Q, 32 kDa and 34 kDa for pro-Der p 1-N52Q, 32 kDa for pro-Der f 1-N53Q, 40–50 kDa for mature Der p 1-WT and Der f 1-WT, and 50–70 kDa for pro-Der p 1-WT and pro-Der f 1-WT.

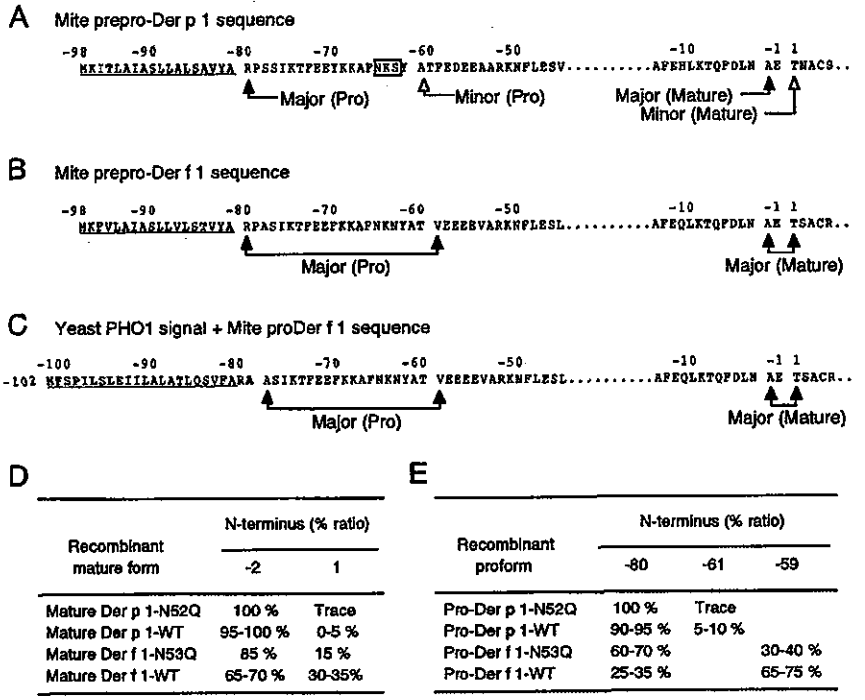
The mobilities on SDS–PAGE of mature Der p 1-N52Q and Der f 1-N53Q were compared with those of natural Der p 1 and Der f 1 (Fig. 1C). The mobilities of the recombinant mature forms without the N-glycosylation expressed in yeast were equivalent to those of the natural allergens purified from mites.

3.3. *N-terminal sequencing of recombinant pro- and mature forms of Der p 1 and Der f 1*

The samples of purified recombinant pro- and mature forms of Der p 1 and Der f 1 were electrophoresed and electroblotted, and the bands visualized by CBB-staining were subjected to N-terminal sequencing. The results are summarized in Fig. 2.

The N-terminal sequences of the four mature forms (Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT) were determined to start from −2 or 1, two-residues upstream from or at the N-terminus of the natural ones (Fig. 2A,B). However, the ratios of the peaks corresponding to these two sequences in the four recombinant variants were different from one another (Fig. 2D). The contents of the peaks corresponding to the sequence starting from the N-terminus of the natural ones were only trace amounts in Der p 1-N52Q and minor amounts in Der p 1-WT.

The N-terminal sequences of the two proforms of Der p 1



D

Recombinant mature form	N-terminus (% ratio) -2	1
Mature Der p 1-N52Q	100 %	Trace
Mature Der p 1-WT	95-100 %	0-5 %
Mature Der f 1-N53Q	85 %	15 %
Mature Der f 1-WT	65-70 %	30-35%

E

Recombinant proform	N-terminus (% ratio) -60	-61	-59
Pro-Der p 1-N52Q	100 %	Trace	
Pro-Der p 1-WT	90-95 %	5-10 %	
Pro-Der f 1-N53Q	60-70 %		30-40 %
Pro-Der f 1-WT	25-35 %		65-75 %

Fig. 2. N-terminal amino acid sequencing of purified recombinant pro- and mature forms of the major house dust mite group 1 allergens Der p 1 and Der f 1. Schematic representation of N-terminal amino acid residues of recombinant pro- and mature forms of Der p 1 in this study (A), Der f 1 in this study (B), and Der f 1 in our previous report [35] (C), and approximate molecular ratios of variations in the different N-terminal sequences of the mature forms (D) and the proforms (E) are shown. In A and B, mite-derived original amino acid sequences were used. In C, yeast *Pichia*-derived PHO1 signal sequence was fused to the mite prosequence. The bold type indicates mutation as a result of introducing a restriction site, and the sequence in the previous report [35] is revised here. Underlined: putative signal sequence for secretion; boxed: N-glycosylation motif. In D and E, the ratios were approximately estimated from the peak heights of HPLC in the protein sequencing. N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence; Trace: peaks corresponding to the sequence starting from the N-terminus were detected as only a trace.

(pro-Der p 1-N52Q and pro-Der p 1-WT) were determined to mainly start from −80, which was just after the putative signal-cleavage site in the original mite prepro-Der p 1 sequence (Fig. 2A,E). Peaks corresponding to the sequence starting from −61, which was 19 residues downstream from the putative signal-cleavage site and 4 residues downstream from the N-glycosylation site in the prosequence of Der p 1, Asn (−65), were also observed but there were only trace amounts in pro-Der p 1-N52Q and minor amounts in pro-Der p 1-WT. The N-terminal sequences for the two major bands for pro-Der p 1-N52Q corresponding to 31 kDa and 33 kDa on SDS–PAGE under non-reducing conditions, or 32 kDa and 34 kDa under reducing conditions, were identical.

The N-terminal sequences of the proforms of Der f 1 (pro-Der f 1-N53Q and pro-Der f 1-WT) were determined to start from −80, which was just after the putative signal-cleavage site in the original mite prepro-Der f 1 sequence, and also from −59, which was 21 residues downstream from the putative signal-cleavage site (Fig. 2B,E).

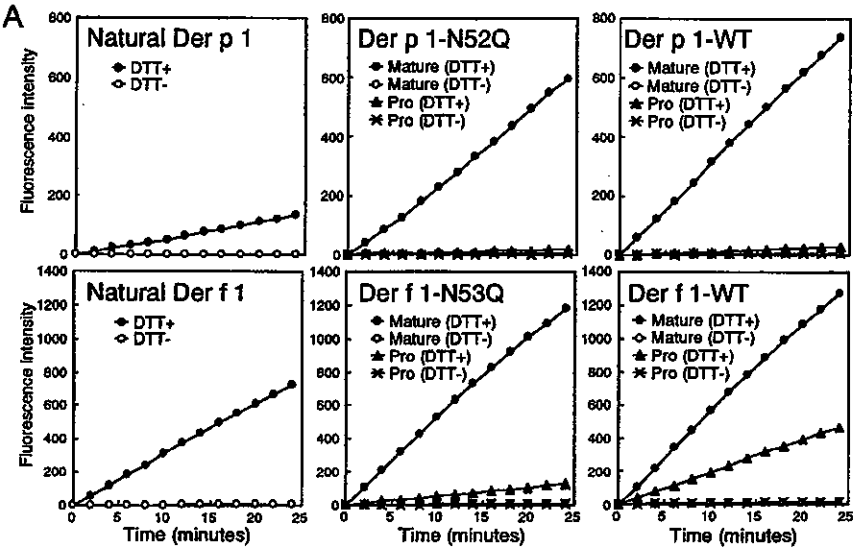
3.4. Cysteine protease activities of natural and recombinant forms of Der p 1 and Der f 1

The proteolytic activities of a total of ten variants of purified natural forms and recombinant pro- and mature forms of Der p 1 and Der f 1 were analyzed in the presence or absence of DTT, which activates the catalytic site of cysteine proteases (Fig. 3A). The natural allergens (natural Der p 1 and natural Der f 1) and the four recombinant mature forms (Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT) retained their activities in the presence of DTT but did not in the absence of DTT. Those of the two proforms of Der p 1 (pro-Der p 1-N52Q and pro-Der p 1-WT) were very low in the presence of DTT and not detectable in the absence of DTT. The two proforms of Der f 1 (pro-Der f 1-N53Q and pro-Der f 1-WT) retained higher protease activities than the proforms of Der p 1, although they were immature compared with their mature forms in the presence of DTT (Fig. 3B), and showed no activities in the absence of DTT.

3.5. IgE-binding of natural and recombinant mature forms of Der p 1 and Der f 1

Patient IgE-binding profiles of recombinant mature forms of Der p 1 and Der f 1 were determined by RAST-EIA (Fig. 4). In sera from two mite-allergic patients, the recombinant mature Der p 1 (Der p 1-WT and Der p 1-N52Q) and Der f 1 (Der f 1-WT and Der f 1-N53Q) with or without the N-glycosylation showed IgE-binding curves equivalent to those of natural Der p 1 and Der f 1, respectively (Fig. 4A). Using thirty or twenty-seven individual sera from mite-allergic patients, IgE-binding to recombinant Der p 1-N52Q or Der f 1-N53Q, which lacked the yeast-derived N-glycosylation, was compared with that of natural Der p 1 or Der f 1 purified from mites, respectively (Fig. 4BC). Close correlations of IgE-binding between natural Der p 1 and recombinant Der p 1-

A



B

Recombinant	Relative proteolytic activity to mature Der f 1-WT (%)
Mature Der p 1-N52Q	47
Mature Der p 1-WT	58
Mature Der f 1-N53Q	93
Mature Der f 1-WT	100
Pro-Der p 1-N52Q	1
Pro-Der p 1-WT	2
Pro-Der f 1-N53Q	10
Pro-Der f 1-WT	37

Fig. 3. Proteolytic activities of purified recombinant and natural types of the major house dust mite group 1 allergens Der p 1 and Der f 1. A: Time courses of digestion of Boc-Gln-Ala-Arg-MCA. The values are calculated by subtracting the background of the substrate, and the initial values from the original values are shown. B: Relative proteolytic activities of the recombinant proforms to that of Der f 1-WT. The values are calculated from fluorescence intensities in the presence of DTT at 24 min. N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence; mature: mature form; pro: proform; DTT+ and DTT-: the reaction was performed in the presence and absence of DTT, respectively.

N52Q ($R^2 = 0.9805$, $P < 0.0001$) (Fig. 4B) and between natural Der f 1 and recombinant Der f 1-N53Q ($R^2 = 0.9877$, $P < 0.0001$) (Fig. 4C) were observed.

4. Discussion

The first reports of activation of a recombinant house dust mite group 1 allergen were made by Shoji et al. [45,46], in which recombinant pro-Der f 1 secreted by insect SF9 cells was converted to active prosequence-removed Der f 1 by dialysis against an acidic buffer. Recently, we also demonstrated that proforms of Der f 1 secreted by yeast *P. pastoris* cells matured to the enzymatically active mature forms [35]. However, concerning Der p 1, recombinant forms of pro-Der p 1 secreted by insect *Drosophila* cells [38] and mammalian CHO cells [39] were not convertible to enzymatically active mature forms and, very recently, van Oort et al. [40] reported that pro-Der p 1 secreted by *P. pastoris* with yeast-derived heterogeneous sugar chains was not spontaneously processed, while Jacquet et al. [41] reported the activation of the highly glycosylated form. Some molecular mechanisms might underlie such discrepancies between the results in these trials to obtain active recombinant forms of Der f 1 and Der p 1. Therefore, the purposes of our study were not only to establish a system for the preparation of active Der p 1 with properties equivalent to natural Der p 1, but also to provide some insights into the molecular mechanisms in processing, activation, and/or inhibition of maturation of the proforms of the two house dust mite group 1 allergens by comparing the differences in properties among the recombinant products.

We used completely mite-derived amino acid sequences of prepro-Der p 1 and prepro-Der f 1 for the expression in this study (Figs. 1A, 2A,B), while a *Pichia*-derived signal sequence was used in our previous report [35] for secretion of pro-Der f 1 (Fig. 2C). The N-glycosylation of the wild-type mature sequences of both Der p 1 and Der f 1 in *Pichia* cells leads to heterogeneous glycosylation (Fig. 1B, Der p 1-WT, Der f 1-WT). Pro-Der p 1-N52Q without N-glycosylation in the mature sequence showed two major bands on SDS–PAGE (Fig. 1B, Der p 1-N52Q, Pro). This is considered a result of differential modifications of the N-glycosylation motif in the Der p 1 prosequence (Figs. 1A, 2A), which does not exist in the Der f 1 prosequence, on the basis of the results of N-terminal sequencing (Fig. 2E), SDS–PAGE showing a single band for the mature form (Fig. 1B, Der p 1-N52Q, Mature), and analysis of the amino acid compositions (data not shown). Signifi-

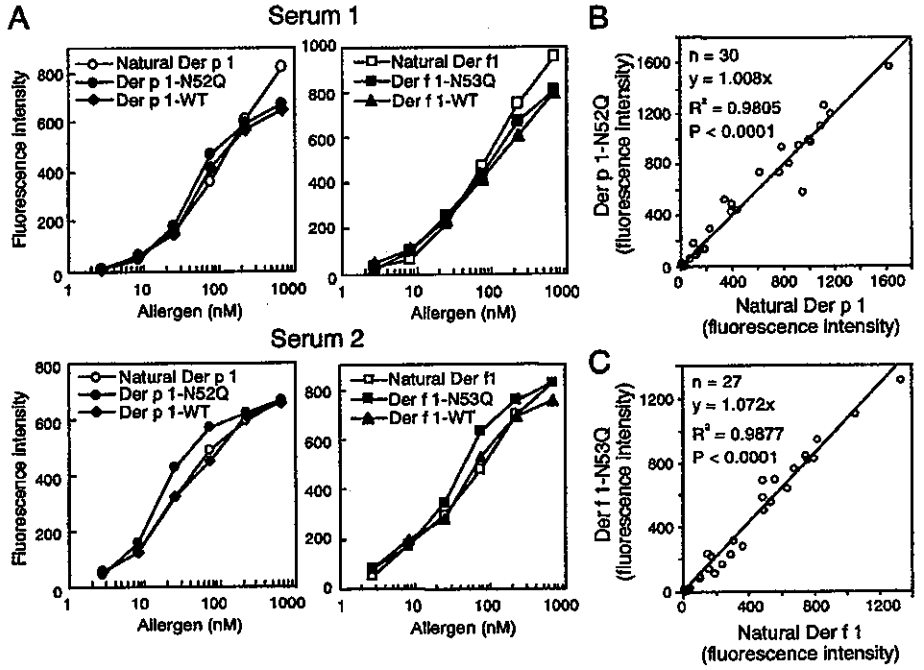


Fig. 4. IgE-binding activities of recombinant mature forms and natural types of the major house dust mite group 1 allergens Der p 1 and Der f 1. Paper discs to which allergen was coupled were incubated with diluted mite-allergic patients' sera and IgE that bound to the discs was detected with β-galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence. A: IgE-binding profiles of recombinant mature forms with and without yeast-derived N-glycosylation, and natural types of Der p 1 and Der f 1 in two sera. B: Correlation between the IgE reactivity of recombinant mature Der p 1-N52Q without yeast-derived N-glycosylation and natural Der p 1. Thirty sera were tested. The concentration of the allergens in the immobilization step was 75 nM. C: Correlation between the IgE reactivity of recombinant mature Der f 1-N53Q without yeast-derived N-glycosylation and natural Der f 1. Twenty-seven sera were tested. The concentration of the allergens in the immobilization step was 25 nM. N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence. The values calculated by subtracting the background of the paper discs without coupling of allergen from the original values are shown.

cantly higher mobility of the mature forms on SDS–PAGE under non-reducing conditions than under reducing conditions is considered a result of the formation of intramolecular disulfide bonds of Der p 1 and Der f 1 [36] (Fig. 1B, Mature). In the mature forms, almost all of the prosequence was removed (Fig. 2A,B,D), and both cysteine protease (Fig. 3A) and IgE-binding (Fig. 4) activities were fully retained. The activated recombinant mature forms are considered to be folded and biologically functional. To obtain recombinant proteins more closely resembling natural ones, we generated mutants in which the N-glycosylation motif within the mature sequence was disrupted. The mutants showed no smear bands on SDS–PAGE (Fig. 1B, Der p 1-N52Q, Der f 1-53Q) and the mature forms of the mutants retained an apparent molecular weight equivalent to the natural ones (Fig. 1C). These recombinant mature house dust mite group 1 allergens with the same properties as the natural types will be useful tools for diagnosis, standardized AIT [18–20], and various other studies, as well as the major house dust mite group 2 allergen from *D. farinae*, Der f 2, which we have investigated as a model allergen for allergen engineering [21–29].

All the visible bands stained after electroblotting were subjected to N-terminal sequencing and variation in the N-terminal sequences existed in the purified recombinant samples (Fig. 2A,B,D,E). Interestingly, the proforms of Der p 1 and Der f 1 differed in the N-terminal sequences (Fig. 2A,B,E) and the proteolytic activity (Fig. 3). While the N-termini of the proforms of Der p 1 (pro-Der p 1-WT and pro-Der p 1-N52Q) mainly started just after the putative signal-cleavage site, the proforms of Der f 1 (pro-Der f 1-WT and pro-Der f 1-N53Q) had those starting not only from the equivalent site but 21 residues downstream (Fig. 2A,B,E), and retained higher activity than the proforms of Der p 1 (Fig. 3). The ratios of partial digestion before position −61 in pro-Der p 1 or −59 in pro-Der f 1 correlated with the proteolytic activities of the proforms (Fig. 2E, Fig. 3B). These results raised two questions. Firstly, why did the difference in the ratio of the partial digestion correlate with the enzymatic activity of the proforms? Secondly, why did the proforms of Der f 1 show higher ratios of partial digestion than those of Der p 1?

The prosequence of protease is considered to help in correct folding of the precursor suitable for mature folding of the prosequence-removed active protease, and to maintain the protease inactive until transfer and exposure to sites or situations where the protease could be sorted and function. As an answer to the first question, we consider that the N-terminal regions of Der p 1 and Der f 1 have a role in blocking the proteolytic activity effectively and/or that removal of the 18 or 20 amino acid sequence in the prosequences of Der p 1 or Der f 1 leads to partial activation of the proforms. This consideration correlates well with recent reports by two groups on the maturation of pro-Der p 1 with the yeast-derived heterogeneous sugar chain secreted by *P. pastoris*, although their results were different from each other, i.e., van Oort et al. [40]

reported that the pro-Der p 1-WT with N-terminus just after the putative signal-cleavage site was mainly obtained and not spontaneously processed, while Jacquet et al. [41] reported that the pro-Der p 1-WT with N-terminus of 19 residues downstream of the putative-cleavage site was obtained and successfully processed to the active mature form. Our results showing that pro-Der p 1-N52Q and pro-Der p 1-WT with the N-terminus just after the putative signal-cleavage site were mainly obtained and processed to the active mature forms was intermediate between those by the two groups. The differences among the three studies could be attributable to differences in procedures for maturation and/or origins of the signal sequences fused to the proforms for secretion.

As an answer to the second question, we suspect that N-glycosylation of the Der p 1 prosequence might be a reason for the higher ratios of partial digestion in pro-Der f 1 than in pro-Der p 1. Interestingly, the minor N-terminus of the proforms of Der p 1 in this study, Ala (−61), is only 4 residues downstream from the N-glycosylation site in the Der p 1 prosequence, Asn (−65) (Fig. 1A, Fig. 2A). Therefore, digestion near Asn (−65) might be inhibited by the sugar chain attachment in pro-Der p 1 differing from pro-Der f 1. Effects of the sugar chain in the prosequence on folding and/or interaction between the prosequence and mature portion might also be possible. This hypothesis could also explain the difficulty in preparing enzymatically active recombinant mature Der p 1 compared to Der f 1 in the previous reports [38–40]. Molecular mechanisms of processing, activation, and/or inhibition of maturation of the proforms should be analyzed in further studies.

In this study, we demonstrated for the first time the preparation of recombinant mature Der p 1, Der p 1-N52Q, which has no yeast-derived N-glycosylation and retains an apparent molecular weight equivalent to natural Der p 1, with full enzymatic and IgE-binding activities. The recombinant mature house dust mite group 1 allergens prepared in this study with the same properties as the natural types will be the basis of various studies. Moreover, by comparing the properties of the recombinant pro- and mature forms of the two homologous allergens Der p 1 and Der f 1, we suggested the contribution of the N-terminal region of the Der p 1 prosequence including the N-glycosylation site, Asn (−65), on effective inhibition of proteolytic activity in pro-Der p 1. This region should be a target candidate for the manipulation of proteolytic activities of recombinant variants. Such variants are considered useful as allergen vaccines because the proteolytic activity of Der p 1 has been proposed to be involved in the pathogenesis of allergies [13–17,30]. The findings in this study implicate a method for effective blockade of the harmful proteolytic activity of the major house dust mite group 1 allergens.

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D-Pro^2-Endomorphin-1 and D-Pro^2-Endomorphin-2, Respectively, Attenuate the Antinociception Induced by Endomorphin-1 and Endomorphin-2 Given Intrathecally in the Mouse

KUEI-CHUN HUNG, HSIANG-EN WU, HIROKAZU MIZOGUCHI, SHINOBU SAKURADA, TORU OKAYAMA, TSUTOMU FUJIMURA, KIMIE MURAYAMA, TSUKASA SAKURADA, JAMES M. FUJIMOTO, and LEON F. TSENG

Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin (K.-C.H., H.-E.W., H.M., J.M.F., L.F.T.); Department of Physiology and Anatomy, Tohoku Pharmaceutical University, Sendai, Japan (S.S.); Research Institute, Fuji Chemical Industries Ltd., Takaoka, Japan (T.O.); Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo, Japan (T.F., K.M.); and Department of Biochemistry, Daiichi College of Pharmaceutical Science, Fukuoka, Japan (T.S.)

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ABSTRACT

First, the antinociception with the tail-flick test of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 given i.t. was compared with that produced by endomorphin-1 and -2 in male CD-1 mice. High doses of D-Pro^2-endomorphin-1 (0.2–0.4 pmol) and D-Pro^2-endomorphin-2 (300–800 pmol) given i.t. produced antinociception with low intrinsic activity [about 25% maximum possible effect (MPE)] compared with that of endomorphin-1 (16.4 nmol) and endomorphin-2 (35 nmol) (>90% MPE). Second, coadministration of a low dose of D-Pro^2-endomorphin-1 (0.1 pmol), which given alone did not affect the tail-flick latencies, markedly attenuated the antinociception induced by endomorphin-1 (16.4 nmol) but not by endomorphin-2 (35 nmol). Similarly, coadministration of a low dose of D-Pro^2-endomorphin-2 (200 pmol), which given alone did not affect the tail-flick latencies, significantly attenuated the antinociception induced by endomorphin-2 (35 nmol) and, to a much lesser extent, endomorphin-1 (16.4 nmol). It is concluded that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 at high doses were partial opioid receptor agonists to produce antinociception, and at low doses were opioid receptor antagonists to block selectively the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Furthermore, our results are consistent with the view that the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of μ-opioid receptors.

Endomorphin-1 and endomorphin-2 are two endogenous tetrapeptides isolated from the bovine frontal cortex (Zadina et al., 1997) and human brain (Hackler et al., 1997). These peptides are the first endogenous peptides to be proposed to have high affinity and selectivity for μ-opioid receptors. Receptor-binding assays and immunocytochemical studies reveal that endomorphin-1 and endomorphin-2 potently compete with μ_1- and μ_2-receptors and that they are widely located at the sites in the brain and spinal cord abundant in μ-opioid receptors (Martin-Schild et al., 1997, 1998, 1999; Goldberg et al., 1998; Pierce et al., 1998; Shreff et al., 1998; Wu et al., 1999). Through the stimulation of μ-opioid receptors, endomorphin-1 and endomorphin-2 inhibit the electrical activity of rostral ventrolateral medulla neurons or spinal substantia gelatinosa neurons (Chu et al., 1999; Wu et al., 1999). The release of endomorphin-2 from the spinal cord can be achieved by electrical stimulation (Williams et al., 1999). The administration of endomorphin-1 and endomorphin-2 given i.c.v. and i.t. produces potent antinociceptive responses that are blocked by μ-opioid receptors antagonists naloxone, β-funaltrexamine, and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) (Zadina et al., 1997; Stone et al., 1997; Narita et al., 1998; Tseng et al., 2000). Neither endomorphin-1 nor endomorphin-2 activates μ-opioid receptor-coupled G proteins in μ-opioid receptor knockout mice, and the antinociception induced by endomorphin-1 and endomorphin-2 is attenuated in heterozygous knockout mice and virtually abolished in homozygous knockout mice (Mizoguchi et al., 1999). These findings support the view that antinociception induced by the endomorphin-1 and endomorphin-2 is mediated by the stimulation of μ-opioid receptors.

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ABBREVIATIONS: CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; β-FNA, β-funaltrexamine; nor-BNI, nor-binaltorphimine; MPE, maximum possible effect; ANOVA, analysis of variance; D-Pro-EM-1, D-Pro^2-endomorphin-1; D-Pro-EM-2, D-Pro^2-endomorphin-2.

However, more recent results illustrate that different subtypes of μ-opioid receptors may be involved in antinociceptive effects induced by endomorphin-1 and endomorphin-2. For example, Sakurada et al. (1999) reported that μ_1-opioid receptor antagonist naloxonazine was more effective in blocking the antinociceptive effects induced by endomorphin-2 than endomorphin-1 in mice. The antinociception induced by endomorphin-1 is blocked by μ-opioid receptor antagonists CTOP or β-funaltrexamine (β-FNA) but not by κ-opioid antagonist nor-binaltorphimine (nor-BNI). On the other hand, the antinociception induced by endomorphin-2 is blocked by CTOP or β-FNA and also by nor-BNI (Tseng et al., 2000; Ohsawa et al., 2001). The findings are taken to indicate that two different subtypes of μ-opioid receptors are involved in endomorphin-1- and endomorphin-2-induced antinociception.

D-Pro^2-Endomorphins, analogs of endomorphins containing D-amino acid isomers, were designed to examine whether these analogs produce antinociception after i.t. administration. Also, the ability of the analogs to modify antinociception induced by i.t. endomorphin-1 and endomorphin-2 was investigated.

Materials and Methods

Animals. Male CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA), weighing 25 to 30 g were used. Animals were housed five per group in a room maintained at 22 ± 0.5°C with an alternating 12-h light/dark cycle. Food and water were available ad libitum.

Drugs. Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) were purchased from Calbiochem (La Jolla, CA). The peptides were dissolved in sterile saline solution (0.9% NaCl solution) containing 10% hydroxypropyl-β-cyclodextrin for i.t. injection. D-Pro^2-Endomorphin-1 (Tyr-D-Pro-Trp-Phe-NH_2) and D-Pro^2-endomorphin-2 (Tyr-D-Pro-Phe-Phe-NH_2) were obtained from Dr. T. Sakurada (Department of Biochemistry, Daiichi College of Pharmaceutical Science, Fukuoka, Japan). These D-Pro^2-endomorphins were first completely dissolved in dimethyl sulfoxide and then 0.9% sodium chloride solution was added to a final concentration of dimethyl sulfoxide at 1%.

Assessment of Antinociceptive Response. The antinociceptive response was assessed with the thermal tail-flick test (D'Amour and Smith, 1941). Mice were gently held with the tail positioned in the tail-flick apparatus (model TF6; EMDIE Instrument Co., Maidens, VA) for radiant heat stimulation of the dorsal surface of the tail. The intensity of the heat stimulus was adjusted to cause the animal to flick its tail within 3 to 4 s as the baseline of latency. After measuring the latency, different groups of mice were treated with endomorphin-1, endomorphin-2, or vehicle given i.t., and the tail-flick responses were then measured at different times after injection. The data were expressed as percentage of maximum possible effect (%MPE), which was calculated as $[T_1 - T_0)/(T_2 - T_0)] \times 100$. T_0 and T_1 were predrug and postdrug latency, respectively, and T_2 was the cutoff time that was set at 10 s to minimize tissue damage.

Drug Administration Protocol. The i.t. injection (5 μl) was performed according to the procedure of Hylden and Wilcox (1980) using a 25-μl Hamilton syringe with a 30-gauge needle. Groups of mice were treated i.t. with various doses of endomorphin-1 (0.82–16.4 nmol), endomorphin-2 (1.75–35 nmol), D-Pro^2-endomorphin-1 (0.03–0.4 pmol), D-Pro^2-endomorphin-2 (50–800 pmol), or vehicle, and the tail-flick tests were performed at 2.5, 5, 7.5, 10, 15, and 20 min thereafter. The effects of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 on the tail-flick inhibition induced by endomorphin-1 and endomorphin-2 were studied. Groups of mice were coadministered i.t. with various doses of D-Pro^2-endomorphin-1 (0.03–0.4 pmol) or D-Pro^2-endomorphin-2 (50–800 pmol) with endomorphin-1 (16.4 nmol) or endomorphin-2 (35 nmol) and the tail-flick response was measured thereafter. In another experiment, groups of mice were coadministered i.t. with D-Pro^2-endomorphin-1 (0.1 pmol) or D-Pro^2-endomorphin-2 (200 pmol) with various doses of endomorphin-1 (0.82–16.4 nmol) or endomorphin-2 (1.75–35 nmol), and the tail-flick response was measured thereafter. To establish dose-response curves, at least four doses were used with 8 to 11 mice at each dose. For the calculation of the ED_{50} values for endomorphin-1- and endomorphin-2-induced tail-flick inhibition, the antinociception was assessed using peak effect, which occurred at either 2.5 or 5 min after administration.

Statistical Analysis. The data were expressed as the mean with S.E.M. The maximal %MPE was used to graph dose-response curves for endomorphin-1 and endomorphin-2. GraphPad Prism software (version 3.0; GraphPad Software, San Diego, CA) was used to calculate dose-response curves, ED_{50} values and their confidence intervals. A two-way ANOVA followed by Bonferroni's post test was used to determine the time in which the attenuation of antinociception reached a maximum by D-Pro^2-endomorphin-1 or D-Pro^2-endomorphin-2 administration. A one-way ANOVA followed by Dunnett's test was used to compare the difference for each group versus the control group.

Results

Time Courses and Dose Effects of i.t. Administration of Endomorphin-1, Endomorphin-2, D-Pro^2-Endomorphin-1, and D-Pro^2-Endomorphin-2 on Tail-Flick Response. Groups of mice were injected i.t. with different doses of endomorphin-1, endomorphin-2, D-Pro^2-endomorphin-1, D-Pro^2-endomorphin-2, or vehicle, and the tail-flick response was measured 2.5, 5, 7.5, 10, 15, and 20 min after injection. Intrathecal injection of endomorphin-1 at doses 0.82 to 16.4 nmol (Fig. 1A) and endomorphin-2 at doses 1.75 to 35.0 nmol (Fig. 1B) dose- and time-dependently produced inhibition of the tail-flick response. The inhibition of the tail-flick response induced by endomorphin-1 and endomorphin-2 developed rapidly, reached their peak at 5 min, declined rapidly, and returned to the preinjection level in 20 min. A dose 16.4 nmol of endomorphin-1 or 35 nmol of endomorphin-2 produced about 90% MPE. The antinociceptive ED_{50} values of endomorphin-1 and endomorphin-2 are shown in Table 1. Endomorphin-1 was found to be 2.3-fold more potent than endomorphin-2 to produce the tail-flick inhibition.

D-Pro^2-Endomorphin-1 (0.1–0.4 pmol) (Fig. 2A) and D-Pro^2-endomorphin-2 (200–800 pmol) (Fig. 2B) given i.t. produced an apparent dose-dependent inhibition of the tail-flick response. However, at the three highest doses of each, there was a ceiling effect (about 25% MPE) where the increase in dose did not lead to a greater effect. It seemed that D-Pro^2-endomorphin-1 was about 2000-fold more potent than D-Pro^2-endomorphin-2 to produce the same ceiling effect. A small dose of D-Pro^2-endomorphin-1 (0.1 pmol) or D-Pro^2-endomorphin-2 (200 pmol) did not show any appreciable tail-flick inhibition.

Effects of D-Pro^2-Endomorphin-1 and D-Pro^2-Endomorphin-2 on Tail-Flick Inhibition Induced by Endomorphin-1 and Endomorphin-2, Respectively. The presence of low, ceiling antinociceptive action suggested that these analogs might be acting on the same respective opioid receptors as were endomorphin-1 and endomorphin-2. Then, it might be possible that either a positive or negative effect might be shown for these analogs to modify antinociception produced by endomorphin-1 and endomorphin-2. Groups of

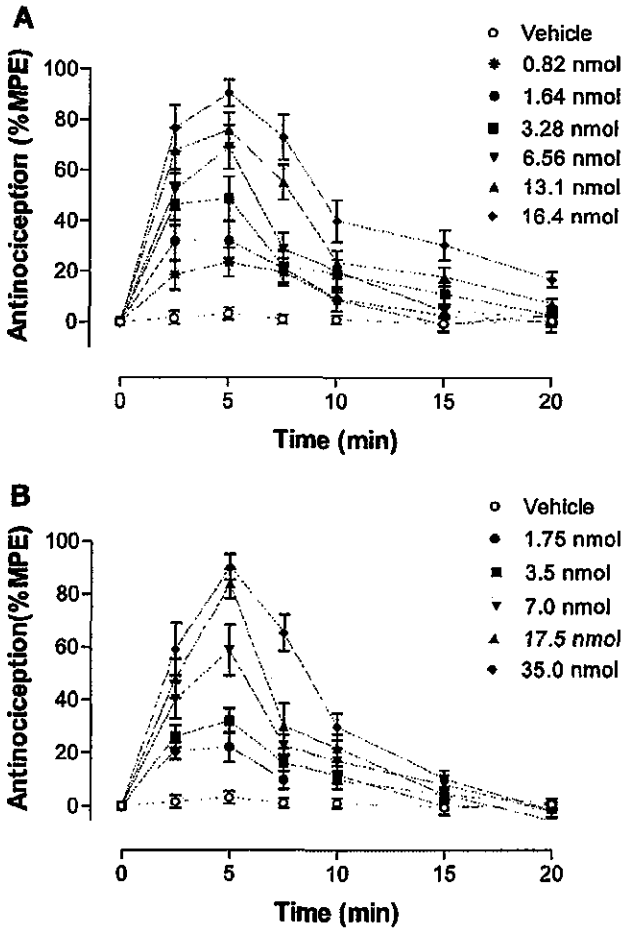

Fig. 1. Time course of changes in the tail-flick inhibition induced by i.t.-injected endomorphin-1 (EM-1; A) and endomorphin-2 (EM-2; B). Groups of mice were injected i.t. with different doses of endomorphin-1, endomorphin-2, or vehicle (5 μl), and tail-flick responses were measured at different times after injection. Each value represents the mean ± S.E.M. with 8 to 12 mice/group.

mice were administered with various doses of D-Pro^2-endomorphin-1 (0.03–0.4 pmol) together with an antinociceptive dose of 16.4 nmol of endomorphin-1. Treatment with D-Pro^2-endomorphin-1 at 0.1 pmol, but not at other higher or lower doses significantly attenuated the tail-flick inhibition induced by endomorphin-1 (Fig. 3A).

A similar experiment was performed for the interaction of D-Pro^2-endomorphin-2 with endomorphin-2. D-Pro^2-Endomorphin-2 at 100, 200, and 300 pmol attenuated the tail-flick inhibition induced by endomorphin-2 (35 nmol) (Fig. 3B).

A crossover experiment then performed with the effective doses of the analogs. As seen in Fig. 3A, 200 pmol of D-Pro^2-endomorphin-2 had a significant effect to reduce the tail-flick inhibition induced by endomorphin-1. However, the effect did not seem to be as great as it was against endomorphin-2 (Fig. 3B). When D-Pro^2-endomorphin-1 was evaluated against endomorphin-2-induced tail-flick inhibition, it had no significant effect (Fig. 3B). Thus, the data indicated that there was minimal crossover of the effects of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 relative to their selectivity of action against endomorphin-1- and endomorphin-2-mediated antinociception.

In the preceding set of experiments, the analogs were given at the same time as endomorphin-1 and endomorphin-2. In the experiment given in Fig. 4, A and B, the time of treatment with the analogs was given 0 (together), 5, and 10 min separately before the endomorphin-1 and endomorphin-2, and tail-flick responses were then measured after the injection. In each case, the respective analogs were most effective when each was given at the same time (0 min) as the endomorphin-1 and endomorphin-2.

Effects of i.t. Treatment with a Fixed Dose of D-Pro^2-Endomorphin-1 and D-Pro^2-Endomorphin-2 on Dose-Response Curves for i.t. Endomorphin-1- and Endomorphin-2-Induced Tail-Flick Inhibition. Endomorphin-1 at doses 0.82 to16.4 nmol or endomorphin-2 at doses 1.75 to 35 nmol given i.t. dose-dependently inhibited the tail-flick response. The ED_{50} values, Hill slope function, and their 95% confidence intervals are given in Table 1. Intrathecal coadministration of 0.1 pmol of D-Pro^2-endomorphin-1 with endomorphin-1 markedly attenuated the antinociceptive response induced by endomorphin-1; the dose-response curve for endomorphin-1 was shifted to the right by 5.5-fold. The shift of the dose-response curve for endomorphin-1-induced tail-flick inhibition after D-Pro^2-endomorphin-1 was not significantly deviated from parallel (Fig. 5A; Table 1). Similarly, i.t. coadministration of 200 pmol of D-Pro^2-endomorphin-2 with endomorphin-2 markedly attenuated the antinociceptive response induced by endomorphin-2; the dose-response curve for endomorphin-2 was shifted to the right by 3.9-fold. The shift of the dose-response curve for endomorphin-2-induced tail-flick inhibition after D-Pro^2-endomorphin-2 was not significantly deviated from parallel (Fig. 5B; Table 1).

Discussion

As in our previous report (Ohsawa et al., 2001), i.t. administration of endomorphin-1 and endomorphin-2 produced antinociception with full intrinsic activity (>90% MPE). Endomorphin-1 was about 2.3- fold more potent than endomoprhin-2. The analogs of endomorphins, D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2, at high doses, also

TABLE 1
The antinociceptive potencies (ED_{50}) and the slope function of dose-response curves for i.t. administration of endomorphin-1 and endomorphin-2 given alone or with D-Pro^2-endomorphin-1 or D-Pro^2-endomorphin-2

	ED_{50} (95% CI)	Hill Slope (95% CI)
	nmol	
Endomorphin-1	2.41 (1.84–3.15)	1.06 (0.74–1.37)
Endomorphin-2	5.60 (4.43–7.09)	1.44 (0.94–1.94)
Endomorphin-1 + 0.1 pmol D-Pro^2-endomorphin-1	13.11 (7.14–24.08)	0.64 (0.23–1.05)
Endomorphin-2 + 200 pmol D-Pro^2-endomorphin-2	21.63 (12.10–38.64)	0.74 (0.36–1.12)

CI, confidence interval.

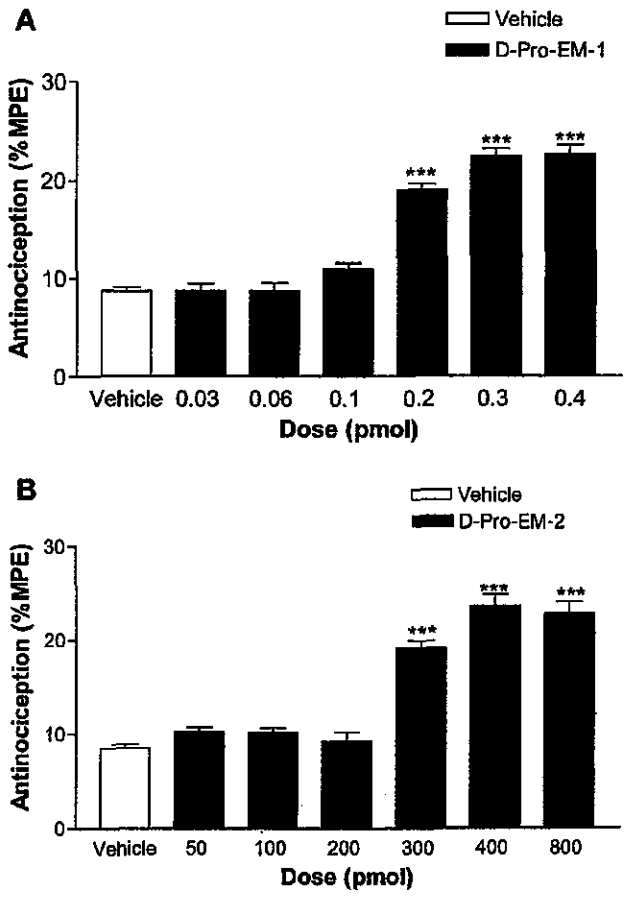

Fig. 2. Tail-flick response after i.t. administration of D-Pro^2-endomorphin-1 (D-Pro-EM-1; A) or D-Pro^2-endomorphin-2 (D-Pro-EM-2; B). Groups of mice were injected i.t. with various doses of D-Pro^2-endomorphin-1 (0.03–0.4 pmol), D-Pro^2-endomorphin-2 (50–800 pmol), or vehicle, and tail-flick responses were measured 2.5, 5, 7.5, 10, 15, and 20 min after administration. Administration of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 dose dependently increased the tail-flick latencies, reached the peaks either 2.5 or 5 min, and declined to the preinjection levels in 20 min after injection. The peak tail-flick inhibition of each mouse, which was occurred either at 2.5 or 5 min after injection, was used to assess the antinociceptive effects of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2. Each column represents the mean ± S.E.M. with 8 to 12 mice/group. A one-way ANOVA followed by Dunnett's test was used to compare the difference for each group versus the vehicle group. ***, $P < 0.001$.

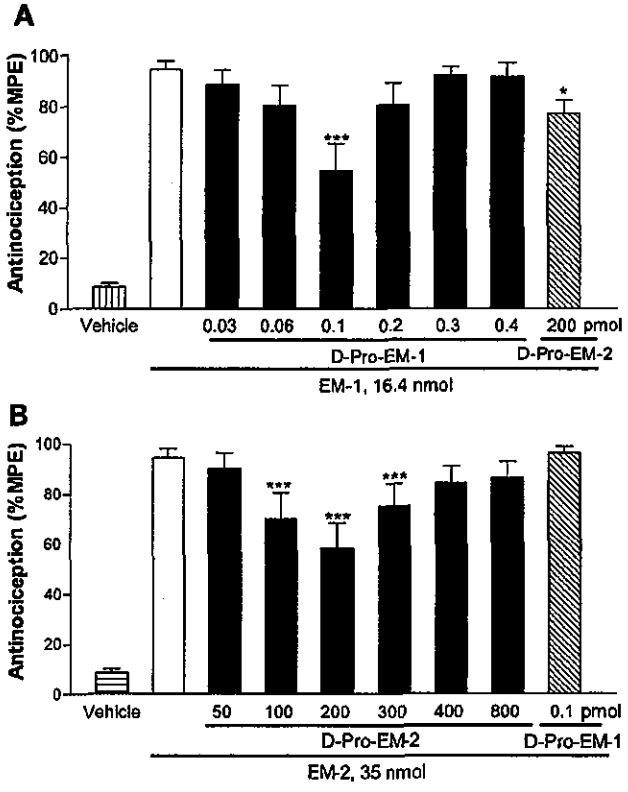

Fig. 3. Effects of i.t. treatment of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 on the inhibition of the tail-flick response induced by endomorphin-1 (A) and endomorphin-2 (B). Groups of mice were treated with different doses of D-Pro^2-endomorphin-1 or D-Pro^2-endomorphin-2 mixed with endomorphin-1 with a final dose of 16.4 nmol or endomorphin-2 with a final dose of 35 nmol. The tail-flick response was measured after the injection. Each column represents the mean ± S.E.M. with 8 to 12 mice/group. A one-way ANOVA followed by Dunnett's test was used to compare the difference for each group versus the control group. *, $P < 0.05$; ***, $P < 0.001$ compared with the mice treated with endomorphin alone.

produced antinociception, but with low intrinsic activity (about 25% MPE) and a ceiling effect. Thus, D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 were partial agonists compared with endomorphin-1 and endomorphin-2. The antinociceptive properties of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 we found in the present studies in mice are consistent with the findings by others in rats (Shane et al., 1999; Krzanowska et al., 2000).

We found in the present studies that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 at small doses are antagonists and antagonized the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Coadministration of D-Pro^2-endomorphin-2 with endomorphin-2 given i.t. attenuated the antinociception induced by endomorphin-2 and only slightly attenuated the antinociception induced by endomorphin-1. These results suggest that D-Pro^2-endomorphin-2 seem to block the μ-opioid receptors predominantly stimulated by endomorphin-2 and to a lesser extent μ-opioid receptors stimulated by endomorphin-1. In addition, coadministration with D-Pro^2-endomorphin-1 with endomorphin-1 attenuated the antinociception induced by endomorphin-1, but not endomorphin-2, indicating that D-Pro^2-endomorhin-1 blocks μ-opioid receptors stimulated by endomorphin-1 only but not by endomorphin-2. The results of our present studies provide additional evidence to support the view that the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of μ-opioid receptors.

D-Pro^2-Endomorphin-1 and D-Pro^2-endomorphin-2 blocked the antinociception induced by endomorphin-1 and endomorphin-2 only at small doses, but not at high doses. Only 0.1 pmol of D-Pro^2-endomorphin and 100 to 300 pmol of D-Pro^2-endomorphin-2, but not higher doses, blocked the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Because D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 at high doses produced a weak antinociception with low intrinsic activity and ceiling effect, the failure for high doses of D-Pro^2-endomorphin-1 or D-Pro^2-endomorphin-2 to block the endomorphin-1- or endomorphin-2-induced antinociception is unexpected. The endomorphin-1 was found to be only

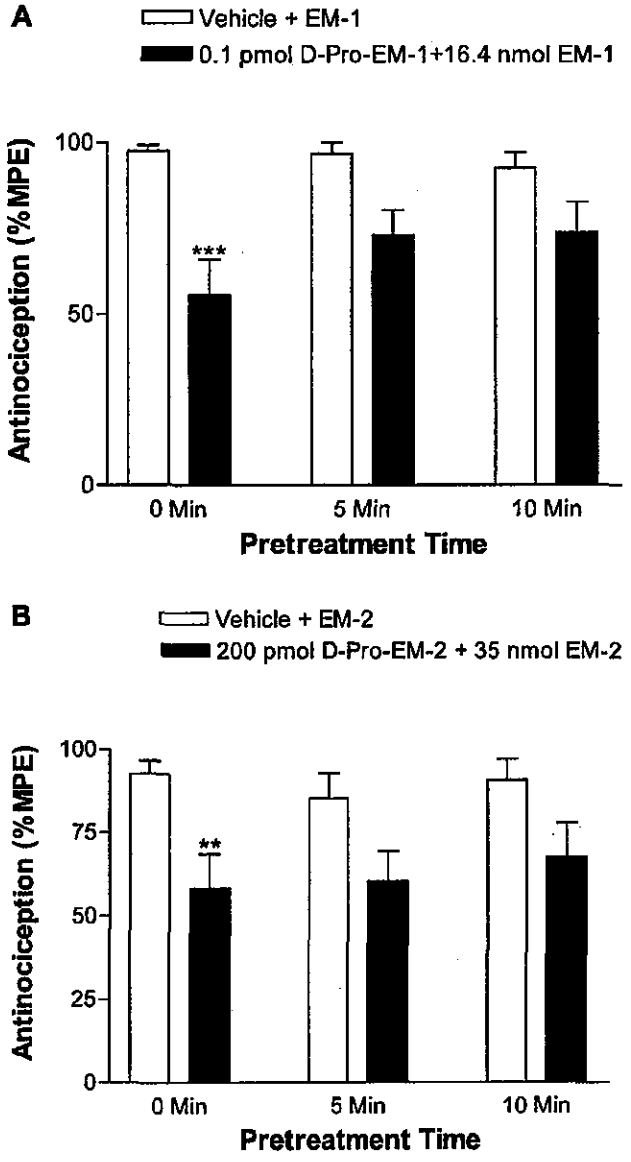

Fig. 4. Time course of i.t. pretreatment with D-Pro^2-endomorphin-1 (A) and D-Pro^2-endomorphin-2 (B) on the inhibition of the tail-flick response induced by i.t. injection of endomorphin-1 and endomorphin-2, respectively. A, groups of mice were pretreated with 0.1 pmol of D-Pro^2-endomorphin-1 or vehicle (5 μl) 0, 5, or 10 min before i.t. administration of endomorphin-1 (16.4 nmol). Tail-flick responses were then measured after each injection. B, groups of mice were pretreated with 200 pmol of D-Pro^2-endomorphin-2 or vehicle (5 μl) 0, 5, or 10 min before i.t. administration of endomorphin-2 (35 nmol), and tail-flick responses were measured after the injection. Each column represents the mean, and the vertical bar represents the S.E.M. with 8 to 11 mice/group. A two-way ANOVA followed by Bonferroni's post test was used to test the difference among groups. **, $P < 0.01$; ***, $P < 0.001$ compared with mice pretreated with the vehicle.

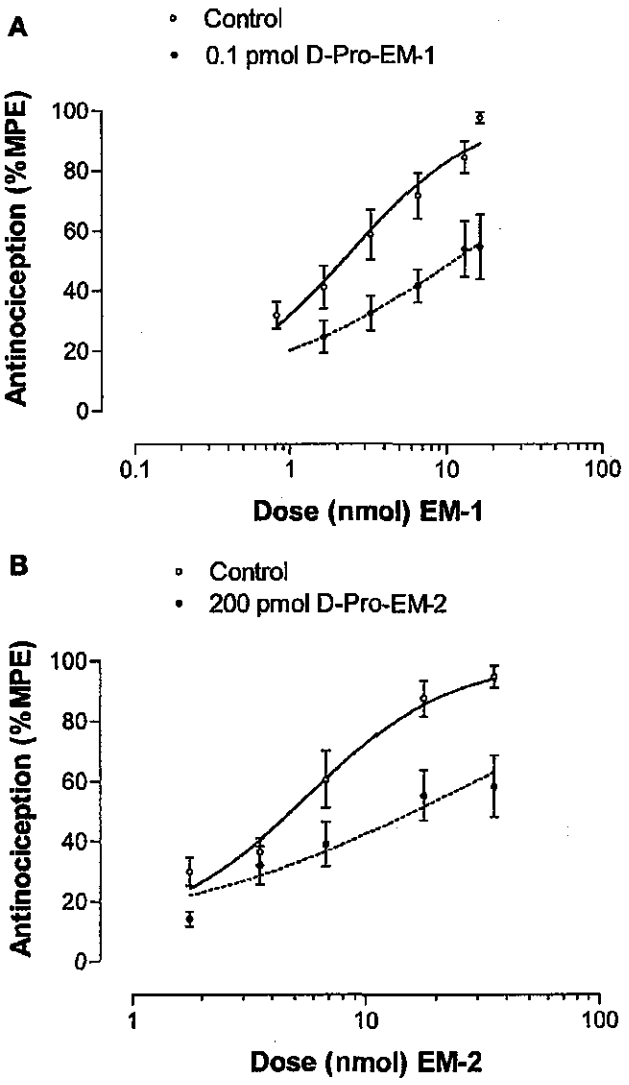

Fig. 5. Effects of i.t. treatment with D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 on dose-response curves for the inhibition of the tail-flick response induced by i.t.-administered endomorphin-1 (A) and endomorphin-2 (B). Groups of mice were simultaneously treated with various doses of endomorphin-1 and 0.1 pmol of D-Pro^2-endomoprhin-1 or various doses of endomorphin-2 and 200 pmol of D-Pro^2-endomorphin-2. Tail-flick responses were measured then after the injection. Each vertical column represents the mean ± S.E.M. with 8 to 12 mice/group.

2.3-fold more potent than endomorphin-2 to produce the antinociception, but D-Pro^2-endomorphin-1 was found to be at least 2000-fold more potent than D-Pro^2-endomorphin-2 to produce the antinociception and to block the antinociception induced by endomorphin-1 and endomorphin-2, respectively (Figs. 2 and 3; Table 1). It is possible that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 may, respectively, bind to different subtypes of μ-opioid receptors to produce agonistic and antagonistic effects.

The view that different subtypes of μ-opioid receptors are involved in endomorphin-1- and endomorphin-2-induced antinociception has been reported (Sakurada et al., 1999, 2000; Ohsawa et al., 2000; Wu et al., 2001). Pretreatment with μ_1-opioid receptor antagonist naloxonazine is more effective in antagonizing the antinociception induced by endomorphin-2 than endomorphin-1. Also pretreatment with 3-methynaltrexone, a morphine-6β-glucuronide antagonist, blocks the antinociception induced by endomorphin-2, but not endomorphin-1 (Sakurada et al., 1999, 2000). A unidirectional cross-tolerance between endomorphin-1 and endomorphin-2 in mice has been reported. Mice made tolerant to endomorphin-1 by i.c.v. pre-

treatment with endomorphin-1 exhibit nearly no cross-tolerance to endomorphin-2 to produce antinociception. On the other hand, mice made tolerant to endomorphin-2 exhibit partial cross-tolerance to endomorphin-1 (Wu et al., 2001). The antinociception induced by endomorphin-1 is blocked by μ-opioid receptor antagonists CTOP or β-FNA but not by κ-opioid antagonist nor-BNI. On the other hand, the antinociception induced by endomorphin-2 is blocked by CTOP, β-FNA, or nor-BNI (Tseng et al., 2000; Ohsawa et al., 2001). Furthermore, i.t. pretreatment with antiserum against dynorphin A(1-17) blocks the antinociception induced by endomorphin-2, but not endomorphin-1. Thus, the antinociception induced by endomorphin-1 and endomoprhin-2 is mediated by the stimulation of different subtypes of μ-opioid receptors. The endomorphin-2-induced antinociception contains an additional component, which is mediated by the spinal release of dynorphin A(1-17) acting on κ-opioid receptors in the spinal cords (Ohsawa et al., 2001).

In opioid receptor binding assay in mouse brain homogenates, both endomorphin-1 and endomorphin-2 compete for both μ_1- and μ_2-receptor binding sites potently, consistent with the view that the antinociception induced by endomorphin-1 and endomorphin-2 is primarily mediated by the stimulation of μ-opioid receptors. However, the study was unable to identify the presence of different subtypes of μ-opioid receptors for endomorphin-1 and endomorphin-2 (Goldberg et al., 1998).

It is concluded that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 at high doses are partial opioid agonists that produce week antinociception and at low doses are antagonists to block selectively the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Our results provide evidence that the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of μ-opioid receptors.

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Address correspondence to: Dr. Leon F. Tseng, Department of Anesthesiology, Medical Education Bldg., Room M4308, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. E-mail: ltseng@mcw.edu

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Characterisation and expression of the *Fasciola gigantica* cathepsin L gene☆

Hiroshi Yamasaki[a,*], Reiko Mineki[b], Kimie Murayama[b], Akira Ito[c], Takashi Aoki[a]

[a]*Department of Parasitology, Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo 113-0033, Japan*
[b]*Division of Biochemical Analysis, Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo 113-0033, Japan*
[c]*Department of Parasitology, Asahikawa Medical College, Asahikawa 078-8510, Japan.*

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Abstract

The gene structure of a cathepsin L from *Fasciola gigantica* was characterised. The gene spans approximately 2.0 kb and comprises four exons and three introns and is a compact gene as in the cases of crustaceous and platyhelminth cathepsins L. Southern blot analysis suggested that a few copies of the genes are sparsely organised in the genome. Of the three intron insertion positions, two of which are in the same position as in the mammalian cathepsin L gene. Phylogenetic analysis revealed that *F. gigantica* cathepsin L forms a clade with those from *Fasciola hepatica*, but not with those from *Spirometra erinacei* and schistosomes. Putative TATA-boxes were found upstream of a transcription initiation site. The sequence analysis of the 5′-upstream of the transcript revealed that the cathepsin L gene is transcribed by *cis*-splicing fashion. Furthermore, the experiments using recombinant *F. gigantica* procathepsin L showed that it was processed to an enzymatically active cathepsin L by pH-dependent autocatalysis. However, the pro-peptide deleted cathepsin L showed no enzyme activity, indicating that the pro-region of *F. gigantica* procathepsin L is essential for the folding and/or refolding of functional cathepsin L. These results are consistent with the observations in mammalian cathepsin L and papain.

Keywords: *Fasciola gigantica*; Cathepsin L; Gene structure; Gene family; Expression; In vitro processing

1. Introduction

The liver flukes *Fasciola hepatica* and *Fasciola gigantica* are causative agents of fascioliasis in humans and ruminants, especially cattle, goat and sheep. The disease has been traditionally considered to be an important veterinary disease because of the substantial production and large economic losses in livestock production. In contrast, human fascioliasis has been linked to cases among livestock in the area concerned. However, it has been considered an increasingly important chronic disease since 1980 (Chen and Mott, 1990; Esteban et al., 1998; Mas-Coma et al., 1999).

It has been reported that *F. hepatica* thiol-activated proteolytic enzymes secreted by migrating parasites cleave host immunoglobulins (Chapman and Mitchell, 1982). Since then, the proteinases secreted by adult liver flukes have been characterised, but most studies agree that the major enzymes are cathepsin L-like cysteine proteases that are homologues of the mammalian lysosomal cathepsin L (Yamasaki et al., 1989; Rege et al., 1989; Dalton and Heffernan, 1989; Fagbemi and Hillyer, 1992; Yamasaki and Aoki, 1993; McGinty et al., 1993; Smith et al., 1993; Wijffels et al., 1994a; Dowd et al., 1994; Heussler and Dobbelaere, 1994; Hawthorne et al., 2000). It is considered that *Fasciola* cathepsin L may be involved in crucial biological functions such as host protein degradation, tissue penetration and immune evasion. For these reasons, the cathepsin L-like cysteine proteases of liver flukes have been potential targets as immunodiagnostic antigens for fascioliasis (Yamasaki et al., 1989; Fagbemi and Guobadia, 1995; O'Neill et al., 1999) or as vaccine candidates (Wijffels et al., 1994a; Dalton et al., 1996b).

On the other hand, concerning genomic organisation of cathepsin L genes in invertebrates, studies on a kinetoplastid protozoon (Eakin et al., 1992), a malaria parasite (Rosenthal and Nelson, 1992), a fruit fly (Matsumoto et al., 1995), a

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* Corresponding author. Present address: Department of Parasitology, Asahikawa Medical College, Midorigaoka Higashi 2-1-1-1, Asahikawa 078-8510, Japan. Tel.: +81-166-68-2421; Fax: +81-166-68-2429.
E-mail address: hyamasak@asahikawa-med.ac.jp (H. Yamasaki).

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larval cestode (Liu et al., 1996) and a shrimp (Boulay et al., 1998) have been reported. In the present paper, we describe the gene structure of a cathepsin L from *F. gigantica* and compare it with those of known cathepsins L from other organisms with a phylogenetic analysis. In addition, the function of the pro-region is also discussed using recombinant *F. gigantica* procathepsin L.

2. Materials and methods

2.1. Parasite

Adult *Fasciola* worms were collected from a cattle naturally infected with the parasites at a local slaughterhouse in Tokyo. The parasites were washed several times in sterile PBS, pH 7.2, and immediately frozen in liquid nitrogen for RNA preparation or stored at −80 °C for DNA samples. The parasite used, *F. gigantica*, was identified based on the nucleotide sequences of cytochrome c oxidase subunit I and ribosomal RNA genes (Hashimoto et al., 1997).

2.2. Construction of the Fasciola genomic DNA library

Genomic DNA was prepared from 1 g of a frozen worm, partially digested with *Sau*3AI, and fractionated into 5–20 kb fragments by sucrose density gradient method (Maniatis et al., 1982). The fragments were ligated to *Bam*HI/*Eco*RI double-digested EMBL 3 lambda DNA, and allowed to form phage particles using an in vitro packaging kit (Stratagene). Approximately 6×10^4 recombinant phages were screened using a digoxigenin-labelled *Fasciola* cathepsin L cDNA probe (Yamasaki and Aoki, 1993).

2.3. Southern blot analysis

Genomic DNA was digested with different restriction enzymes whose cleavage sites are absent from the cathepsin L gene, separated on a 0.7% agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim) and prehybridised at 68 °C for 2 h in hybridisation buffer containing 7% SDS. The filter was hybridised at 68 °C overnight with a digoxigenin-labelled probe. The 1.9-kb probe was prepared by PCR using primers based on the nucleotide sequences of *F. gigantica* cathepsin L gene (B22-2) shown in Fig. 2. Finally, the filter was washed at high stringency and exposed to X-ray film for 5–10 min. CSPD (Boehringer Mannheim) was used as a substrate for chemiluminescent detection.

2.4. RNA preparation, 5′-RACE and Northern blot analysis

Total RNA was extracted from a frozen worm (0.8 g weight) by the acid guanidinium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified on an oligo(dT) cellulose gel column (Pharmacia Amersham Biotech, USA). 5′-rapid amplification of cDNA ends (5′-RACE) was performed according to the manufacturer's instructions (Life Technologies Inc.) to determine the sequence of the 5′-untranslated region of the *F. gigantica* cathepsin L gene (*Fg-CATL*). Gene-specific antisense primers (GSP 1, 5′-TTGATGCCACAAATCATCATTCGAGCCAAG-3′ and GSP 2, 5′-ATCTTCCCAAATATTTCGTCTGTG-3′) were used for nested PCR. PCR was performed for 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, and 4 min at 72 °C for extension. In order to clarify whether a spliced leader sequence is added at the 5′-end of the transcript of *Fg-CATL*, mRNA was reversibly transcribed with M-MLV reverse transcriptase (Gibco BRL) and oligo(dT) primer (Boehringer Mannheim), followed by PCR performed at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, for 35 cycles using a *Fasciola* spliced leader primer (5′-AACCTTAACGGTTCTCTG-3′, Davis et al., 1994) and GSP 1 primer.

mRNA was electrophoresed in a 1% agarose gel, blotted onto a positively charged nylon membrane, and then hybridised with a digoxigenin-labelled cathepsin L (B22-2) cDNA probe overnight at 42 °C. The probe was prepared by PCR using primers based on the nucleotide sequences of *F. gigantica* cathepsin L gene (B22-2) and cDNA prepared using cDNA synthesis kit (Pharmacia). The hybridisation buffer used was 50 mM phosphate buffer, pH 7.0, containing 5× standard saline citrate (SSC), 7% SDS, 0.1% sarcosine, 50% formamide and yeast total RNA (50 μg/ml). The filter was washed extensively with 2× SSC/0.1% SDS, 0.1× SSC/0.1% SDS at 42 °C, and chemiluminescent detection was carried out using CSPD as a substrate.

2.5. DNA sequencing

A DNA fragment derived from a positive clone (B22-2 clone) was digested with restriction enzymes as indicated in Fig. 1, and the resulting fragments were subcloned into pUC18. The nucleotide sequences of the genomic DNA were determined by the dideoxynucleotide chain termination method using Dye Primer and Dye Terminator Cycle Sequencing kits (Applied Biosystems Inc.) and an ABI DNA sequencer 373A. The nucleotide sequence of the PCR-amplified cathepsin L gene (B7-3) was also determined by same protocol. Sequence data were analysed using Genetix-Mac, and EMBL/GenBank database searches were performed with the FASTA program.

2.6. Sequence alignment and phylogenetic analysis

The alignment of sequences was carried out using CLUSTAL W program available over the World Wide Web (http://www.ddbj.nig.ac.jp/E-mail/homology.html). Phylogenetic tree was inferred with the neighbour-joining method (Saitou and Nei, 1987) using TreeView PPC software (version 1.5.3). Confidence values for each branch were determined by 1000 bootstrap replications.