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Assembly of the silk fibroin elementary unit in endoplasmic reticulum and a role of L-chain for protection of α1,2-mannose residues in N-linked oligosaccharide chains of fibrohexamerin/P25

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Silk fibroin of *Bombyx mori* is secreted from the posterior silk gland (PSG) as a 2.3-MDa elementary unit, consisting of six sets of a disulfide-linked heavy chain (H-chain)–light chain (L-chain) heterodimer and one molecule of fibrohexamerin (fhx)/P25. Fhx/P25, a glycoprotein, associates non-covalently with the H–L heterodimers. The elementary unit was found and purified from the endoplasmic reticulum (ER) extract of PSG cells. A substantial amount of fhx/P25 unassembled into the elementary unit was also present in ER. In normal-level fibroin-producing breeds (J-139 and C108), the elementary unit contained fhx/P25 of either 30 kDa (major) or 27 kDa (minor). The 27-kDa fhx/P25 was produced from the 30-kDa form by digestion with the bacterial α1,2-mannosidase *in vitro*. The elementary unit in the ER extract contained only the 30-kDa fhx/P25, whereas both 30- and 27-kDa forms of fhx/P25 were present in the ER plus Golgi mixed extracts. In *naked-pupa* mutants [*Nd(2), Nd-s and Nd-s^D*], extremely small amounts of fibroin were produced and they consisted of one molecule of 27-kDa fhx/P25 and six molecules of H-chain but no L-chain. When the *Nd-s^D* mutant was subjected to transgenesis with the normal *L-chain* gene, the $(H\text{-}L)_6fhx_1$-type elementary unit containing the 30-kDa fhx/P25, was produced. These results suggest that fhx/P25 in the elementary unit is largely protected from digestion with Golgi α1,2-mannosidases when L-chains are present in the unit. Models suggesting a role of L-chain for the protection of α1,2-mannose residues of fhx/P25 are presented.

Keywords: elementary unit of fibroin; fibroin L-chain; fibrohexamerin/P25; transgenic silkworm; Golgi α1,2-mannosidases.

Silk fibroin of the silkworm, *Bombyx mori*, is synthesized in the posterior silk gland (PSG) cells, secreted into the lumen of PSG, associated with sericin in the lumen of the middle silk gland (MSG) and spun out from the anterior silk gland as silk fibers to form a cocoon. The silk fibroin is secreted in a form of a 2.3-MDa protein complex designated as the elementary unit of fibroin [1], which consists of six sets of heavy chain (H-chain; 350-kDa fibrous protein)–light chain (L-chain; 26 kDa) disulfide-linked heterodimer and one molecule of a glycoprotein, fibrohexamerin (fhx)/P25 [2,3]. A single disulfide bond is formed between Cys172 of L-chain and Cys-c20 (20th residue from the C-*termi*nus) of H-chain [4]. Fhx/P25 contains three N-linked oligosaccharide chains at Asn69, Asn113, Asn133 [1] and exists either in a 30-kDa (major) or 27-kDa (minor) molecular form [1,5], which has been suggested to have different compositions of oligosaccharide chains [1]. Fhx/P25 associates with $(H\text{-}L)_6$ mainly by hydrophobic interactions [5] and is centrally important in the maintenance of the elementary unit, because treatment of the elementary unit with 2% Triton X-100, *N*-glycosidase F or endo-H caused partial displacement or deglycosylation of fhx/P25 and simultaneously remarkable disintegration of the elementary unit [1]. On the other hand, treatment of the elementary unit with 1.3 mM dithiothreitol dissociated L-chains completely from H-chains without affecting the gross integrity of the once-formed elementary unit [1]. In the three fibroin-secretion-deficient *naked-pupa* mutant silkworms; *Nd(2)*, *Nd-s* and *Nd-s^D*, the level of secretion of fibroin is less than 1% of that in the normal level fibroin-producing breeds

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Abbreviations: ConA, concanavalin A; ER, endoplasmic reticulum; endo-H, endoglycosidase-H; fhx, fibrohexamerin; H-chain, heavy chain; L-chain, light chain; MSG, middle silk gland; PSG, posterior silk gland.

Enzymes: Bacillus sp. α1,2-mannosidase (EC 3.2.1.24)

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[6,7] and the extremely small amounts of fibroin produced by these three mutant silkworms lack L-chains and show the composition of H_6fhx_1 [1]. The disulfide linkage between H- and L-chains is not formed in *Nd-s* and *Nd-s^D* mutants because of the lack of Cys-172 in the mutant L-chain [8]. It is of interest to note that the fhx/P25 in the secreted fibroin of the three *naked-pupa* mutants is only of the 27-kDa molecular form [5].

In the present study, we aimed at answering the following three specific questions: (a) where is the site of assembly of the elementary unit in PSG cells?; (b) what is the difference between 30-kDa and 27-kDa fhx/P25?; (c) does the L-chain play a role in the formation of the 30-kDa fhx/P25?

Experimental procedures

Breeds of silkworms

Fertilized eggs of *B. mori* J-139 and C108, which are normal-level producers of fibroin, and three fibroin-secretion-deficient *naked-pupa* mutants [*Nd(2)*, *Nd-s* and *Nd-s^D*] were supplied by the National Institute of Agrobiological Sciences, Tsukuba, Japan and their larvae were reared in the authors' laboratories (Tohoku University and National Institute of Agrobiological Sciences). The *Nd(2)* mutation is linked to the fibroin H-chain gene, *fibH* [9]. *Nd-s* and *Nd-s^D* are mutations of the fibroin L-chain gene, *fibL* [8,10].

Preparation of ER or ER plus Golgi extracts from PSG cells

Fifty pairs of PSGs were excised from larvae of *B. mori* J-139 at the fifth day of the fifth instar and layers of giant PSG cells were separated as described [1]. The PSG cell layers were washed in TMK [10 mM Tris/HCl (pH 7.8), 3 mM $MgCl_2$, 150 mM KCl], immersed in 5 mL of TMK containing 0.25 M sucrose and 10 mM phenylmethylsulfonyl fluoride for 30 min at 4 °C, and were forced to pass successively through nylon meshes of 1-mm and 0.1-mm pore sizes. Cells passed through the two nylon meshes were gently disintegrated by passing through a 25-gauge needle six times to yield PSG cell lysate. The lysate was centrifuged at 1000 ***g*** for 5 min at 4 °C to sediment nuclei. The supernatant (2 mL per tube) was layered on 0.4–1.8 M linear sucrose gradient in TMK (12.2 mL per tube), centrifuged at 285 000 ***g*** in a SW40 Ti rotor (Beckman) for 2 h at 4 °C, and 0.5-mL fractions were collected. Fractions containing ER were monitored by the NADPH-cytochrome *c* reductase assay [11] and by Western blotting with the rabbit anti-(*Drosophila* calnexin-peptide) Ig raised against a synthetic peptide, CAQTEESNTKRKRQARKE [12]. For the former assay, a 5-μL portion of each fraction was mixed with 100 μL of the assay mixture [0.08 mM cytochrome *c* (Sigma), 0.1 mM NADPH, 1 mM sodium azide, 10 mM Tris/HCl (pH 7.5)], incubated at 37 °C for 1 min and A_{550} was measured. Fractions containing Golgi complex were monitored by the assay for Golgi α-mannosidase II [13–15] as follows. A 15-μL portion of each fraction was mixed with 135 μL of 0.89 mM 4-metylumbelliferyl-α-D-mannopyranoside (Nacalai Tesque Inc., Kyoto, Japan), 0.22× NaCl/P_i, 0.11% Triton X-100, incubated at 37 °C for 30 min, and then 150 μL of 0.5 M glycine, 0.5 M Na_2CO_3 was added to stop the reaction. The intensity of fluorescence was measured by Fluorescence Spectrometer F-3000 (Hitachi) at the exciting wavelength of 364 nm and the emission wavelength of 448 nm. Fractions containing ER only or ER plus Golgi were pooled, sonicated briefly and used as the ER extract or the ER plus Golgi mixed extracts.

Isolation of the elementary unit of fibroin from the ER extract

The ER extract (about 20 mL) was concentrated to about 1 mL using Molcut II (Millipore). The elementary unit was isolated from the concentrated ER extract by two successive gel filtration column chromatographies as described [1].

Preparation of fibroin and purification of fhx/P25

Fibroin samples from PSG lumen and cocoons were prepared as described [5]. Fhx/P25 was purified from the fibroin secreted into the lumen of PSG of J-139 or partially purified (before the step of reverse-phase HPLC) from following sources; cocoons of *Nd(2)*, *Nd-s*, *Nd-s^D*, C108 or L6 × 7 transgenic line, and protein samples from ER extract or ER plus Golgi mixed extracts of C108 or *Nd-s^D* as described [1].

Antibodies and Western blotting

Western blotting of fibroin samples were carried out as described previously [1] with the following antibodies: the rabbit polyclonal anti-H-chain or the anti-L-chain Ig [1], the mouse polyclonal anti-fhx/P25 Ig [1], the rabbit anti-L28-peptide Ig specific to the normal L-chain [16], the mouse polyclonal anti-(*Nd-s^D*-peptide) Ig specific to the C-terminal sequence of the *Nd-s^D* mutant L-chain [8], or the rabbit anti-(*Drosophila* calnexin-peptide) Ig as described above [12]. The 30- and 27-kDa bands of fhx/p25, detected by Western blotting with the anti-fhx/P25 polyclonal antibody, were subjected to a densitometric analysis using GS-700 Imaging Densitometer (Bio-Rad).

Glycosidase digestion and the reaction with ConA of fhx/P25

Purified fhx/P25 was digested with endo-H (New England Biolabs) under the conditions as described previously [1]. Purified fhx/P25 from J-139 (100 ng) or partially purified fhx/P25 from *Nd(2)*, *Nd-s* or *Nd-s^D* (about 300 ng each) was dissolved in 7 μL of NaCl/P_i (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4), mixed with 7 μL of 1 $\mu g \cdot \mu L^{-1}$ solution of the purified *Bacillus* sp. α1,2-mannosidase (EC 3.2.1.24) [17], and incubated at 37 °C for 12 h. Fibroin samples from cocoons and purified or partially purified fhx/P25 were subjected to SDS/PAGE, transferred to a nitrocellulose membrane, and subjected to the reaction with biotinylated ConA (HONEN). The reaction was detected with alkaline phosphatase-conjugated streptavidin (Gibco-BRL) as described [1].

Quantitative ELISA

Quantification of H-chain, L-chain and fhx/P25 in the elementary unit, PSG cellular fractions or in cocoon proteins was carried out by ELISA with specific antibodies as described [1].

Construction of a recombinant transforming plasmid and production of transgenic silkworms

A 5′-flanking region (−600 to +34) of the *L-chain* gene containing a putative promoter was amplified by PCR from the cosmid clone pKYFL139-2 [18]. The cDNA sequences encoding L-chain and the 3′-flanking region plus a part of poly(A) stretch (+34 to +1354) were amplified by PCR from the cDNA clone pFL18 [19]. These fragments were inserted into pBac(3xP3-DsRed2) transforming vector [20] to construct pBac(3xP3-DsRed2 + L-chain promoter/normal L-chain cDNA) for simultaneous expression of DsRed2 and the normal L-chain. The recombinant plasmid was purified using QIAGEN Midi Kits (Qiagen). Fertilized eggs of the *Nd-s*^{D} mutant silkworm within 4 h after oviposition were treated with 0.9 M HCl for 1 h at 25 °C to break embryonic diapause. Two to three nanoliters of a mixture of the helper plasmid pHA3PIG [21] and the above recombinant plasmid (0.2 $\mu g \cdot \mu L^{-1}$ DNA, each) in 0.5 mM phosphate buffer (pH 7.0), 5 mM KCl was injected into each of the 492 eggs at 4 h after the HCl treatment. Transgenic embryos at the first (G1) or second (G2) generation or moths derived from them were selected by examining the expression of DsRed2 in the stemmata and nervous tissues [22] under Leica MZFL III fluorescence microscope.

Blot hybridization

*Eco*RI-digested genomic DNAs from moths were subjected to Southern blot hybridization with the L-chain 3′-flanking region (from +887 to +1354) from pFL18 [19] as a probe for the normal L-chain gene. Total RNA from PSG or MSG was prepared as described [6,7] and subjected to Northern blot hybridization with following probes, each labeled with AlkPhos Direct Labeling system (Amersham Pharmacia Biotech): a part of the L-chain cDNA sequence (+1 to +357) which is common to normal and *Nd-s*^{D} L-chains amplified by PCR from pFL18 [19] and the *B. mori* elongation factor α-1 isoform cDNA sequence (from +1 to +1682) amplified by PCR from pBmEF-1 α-1 [23].

Results

Assembly of the elementary unit in ER

It has been speculated that the elementary unit of fibroin is assembled in ER and transported to Golgi complex [1] by analogy with other oligomeric secretory proteins [24]. In order to examine this notion, ER was isolated from the PSG cell extract of a normal breed (J-139) by sucrose density gradient centrifugation under nondenaturing conditions (Fig. 1A). Distributions of ER markers (the reaction with anticalnexin-peptide antibody and the NADPH-cytochrome *c* reductase activity) and a Golgi marker (Golgi α-mannosidase II activity) across the gradient indicated that fractions containing sufficiently pure ER, in terms of its separation from Golgi, were obtained (fractions 9–16) but fractions showing activity of the Golgi-marker enzyme (fractions 1–6) still contained substantial ER materials (Fig. 1A). Recentrifugation of fractions 1–4 through the similar sucrose gradient could not yield a sufficiently pure Golgi fraction (data not shown). The H- and L-chains, and fhx/P25 were all present in the ER fraction (fractions 9–16) as detected by specific antibodies.

The peak ER fractions (fractions 9–13 in Fig. 1A) were pooled and subjected to brief sonication to yield an ER extract. The ER extract was then separated on a gel filtration column of TSK-GEL HW75-F, and each fraction was examined by Western blotting with the anti-H-chain, the anti-L-chain, or the anti-fhx/P25 Ig (Fig. 1C). Protein complexes containing H- and L-chains, and fhx/P25 were eluted between fractions 22 and 28, forming two peaks. Because the elution profile of the first peak (fractions 23 and 24, bracketed) was identical, in comparison with the elution profile of β-connectin (2.1 MDa), to that of the elementary unit isolated from PSG cells [1], these fractions were pooled, concentrated, and subjected to re-chromatography on a TSK-GEL HW65-S gel filtration column (Fig. 1D). The protein complex was eluted as a single peak at the position for approximately 2.3-MDa and consisted of H-chain, L-chain, and fhx/P25 as shown by Western blotting (Fig. 1D). When fractions 13–15 (bracketed) were pooled and analyzed by the quantitative ELISA, the molar ratio of H- and L-chains, and fhx/P25 was shown to be close to 6 (H-chain) : 6 (L-chain) : 1 (fhx/P25) (Table 1). Because all these properties are identical to those of the elementary unit isolated from the total PSG cell extract (Table 1 and [1]), we interpreted these results to indicate that the elementary unit of fibroin is assembled in ER.

Fhx/P25 which is not assembled into the elementary unit is present in ER

When the total PSG cell extract was subjected to the quantitative ELISA, the molar ratio of H-chain : L-chain : fhx/P25 was 3.2 : 2.7 : 1 (Table 1). These results suggested that excess fhx/P25 which was not assembled into the elementary unit was present in PSG cells. A similar molar ratio was obtained when the total ER extract was subjected to the quantitative ELISA (Table 1), suggesting that a substantial fraction of fhx/P25 molecules which was not assembled into the elementary unit was retained in ER. This notion was supported by the fact that the lower molecular-mass fraction of the ER extract (fractions 25–32 in Fig. 1C) showed the molar ratio of H-chain : L-chain : fhx/P25 which was similar to that of the total ER extract (Table 1).

The 30- and 27-kDa molecular forms of fhx/P25 are suggested to be caused by the presence or absence of α1,2-mannose residues in the N-linked oligosaccharide chains

It has been shown that fhx/P25 in the secreted fibroin of the normal breed J-139 is either 30-kDa (major) or 27-kDa (minor), whereas only the 27-kDa form is present in the

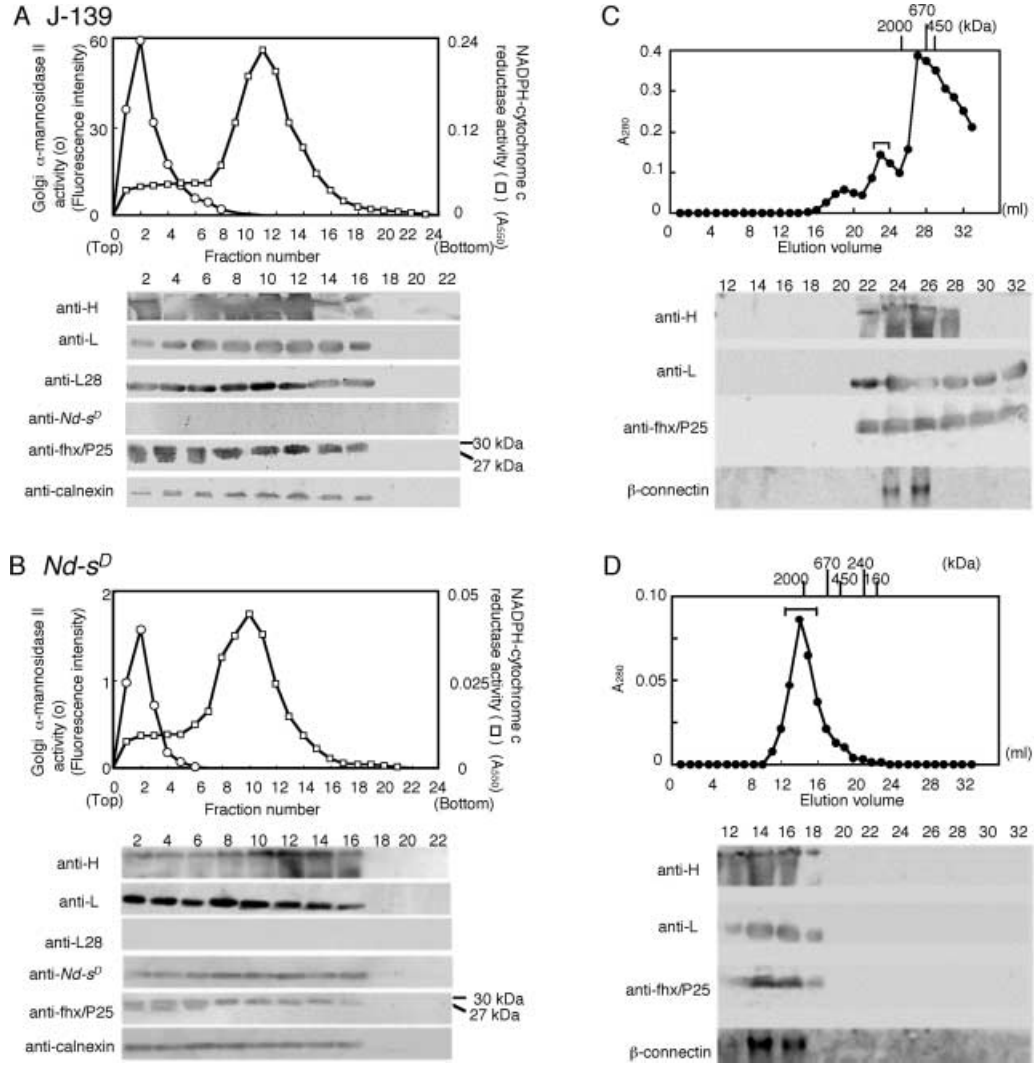

Fig. 1. Fractionation of ER from the PSG cell lysate and purification of the elementary unit of fibroin from the ER extract. The PSG cell lysate from a normal breed J-139 (A) or *Nds*^{D} mutant (B) was subjected to sucrose density-gradient centrifugation. Each fraction was assayed for activities of NADPH-cytochrome *c* reductase (an ER marker) and α-mannosidase II (a Golgi marker). Proteins in every other fraction were analyzed by Western blotting with the anti-H-chain, the anti-L-chain, the anti-L28, the anti-*Nd-s*^{D}, the anti-fhx/P25 or the anti-calnexin-peptide Igs. (C) The ER extract, prepared from pooled fractions 9–13 in (A), was fractionated on a TSK-GEL HW75-F gel filtration column and A_{280} of each fraction was determined. Samples from every other fraction were subjected to SDS/PAGE and Western blotting with the anti-H-chain, the anti-L-chain, or the anti-fhx/P25 Igs. (D) Fractions 23 and 24 from five equivalent columns as in (C) were pooled, concentrated, and subjected to the second gel filtration chromatography on a TSK-GEL HW65-S column and Western blotting as in (C). β-Connectin (2.1 MDa), as a high molecular-mass marker [1], was detected by staining with Coomassie brilliant blue R after 0.1% sodium dodecyl sulfate-2 to 15% gradient PAGE.

Table 1. Determination of molar ratios of H-chain, L-chain and fhx/P25 in different PSG cellular fractions of *B. mori* J-139 (a normal breed). Protein samples in low MW fraction from ER extract were prepared from fractions (25–32) of gel filtration chromatography as shown in Fig. 1(C). $n = 6$; ±, SD.

	Fibroin			Purified elementary unit of fibroin	
	In total cellular extract	In total ER extract	In low MW fraction of ER extract	From total PSG extract	From total ER extract
H-chain	3.24 ± 0.38	3.33 ± 0.36	3.11 ± 0.38	5.94 ± 0.07	5.93 ± 0.16
L-chain	2.72 ± 0.21	2.86 ± 0.25	2.77 ± 0.39	5.80 ± 0.02	6.05 ± 0.09
fhx/P25	1.00	1.00	1.00	1.00	1.00

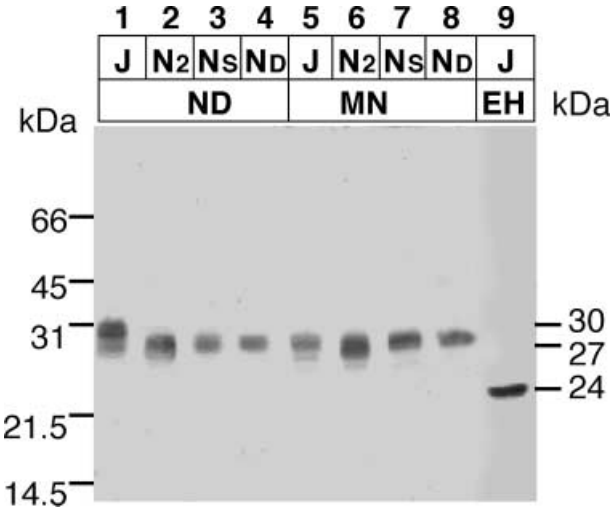

Fig. 2. Effect of digestion with the bacterial α1,2-mannosidase or endo-H on the electrophoretic mobility of fhx/P25. Purified (lanes 1, 5, 9; 100 ng·protein per lane) or partially purified (lanes 2–4, 6–8; 300 ng·protein per lane) preparations of fhx/P25 were subjected to SDS/PAGE and Western blotting with the anti-fhx/P25 antibody before (ND; lanes 1–4) or after (lanes 5–8) digestion with the bacterial α1,2-mannosidase (MN) or after digestion with endo-H (EH; lane 9). Fhx/P25 samples were from a normal breed J-139 (J; lanes 1, 5, 9) or from the fibroin-secretion-deficient *naked-pupa* mutants; *Nd(2)* (N2; lanes 2, 6), *Nd-s* (NS; lanes 3, 7) and *Nd-s*^{D} (ND; lanes 4, 8).

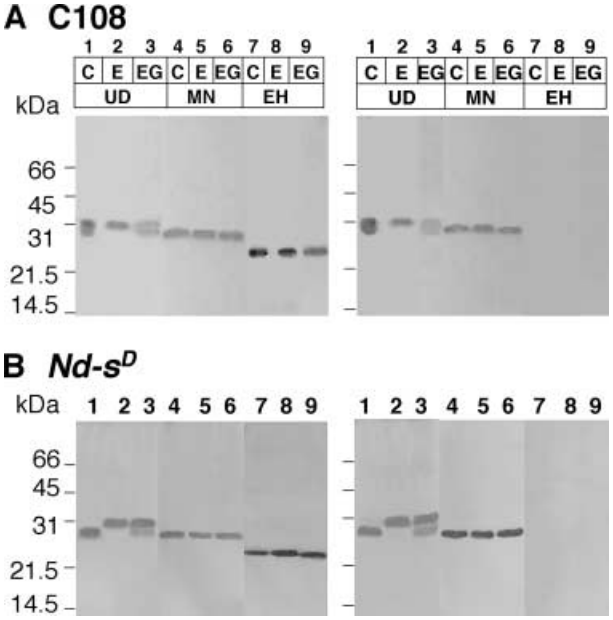

Fig. 3. Different molecular forms of fhx/P25 in cocoons and cellular fractions and digestibility of partially purified fhx/P25 with glycosidases. Fhx/P25 molecules secreted into cocoons (C; lanes 1, 4, 7), present in the ER extract (E; lanes 2, 5, 8), or in the ER plus Golgi mixed extracts (EG; lanes 3, 6, 9) of a normal breed C108 (A) or *Nd-s*^{D} mutant (B) were partially purified, separated by SDS/PAGE and subjected to Western blotting with the anti-fhx/P25 Ig (left panels) or to the lectin blotting with biotinylated ConA (right panels). Protein samples were undigested (UD; lanes 1–3), digested with the bacterial α1,2-mannosidase (MN; lanes 4–6), or digested with endo-H (EH; lanes 7–9).

extremely small amounts of fibroin secreted from the three *naked-pupa* mutants; *Nd(2)*, *Nd-s* and *Nd-s*^{D} [5]. The relative ratio of 30 : 27-kDa forms of fhx/P25 in the purified elementary unit preparation from J-139 was 69.3 (± 10.8) : 30.7 (± 10.8) ($n = 5$) as determined by densitometry after Western blotting. The purified 30-kDa fhx/P25 from J-139 was shown to be converted to the 27-kDa form by digestion with the bacterial α1,2-mannosidase, which cleaves terminal α1,2-linked mannose residues in N-linked oligosaccharide chains. On the other hand, partially purified 27-kDa fhx/P25 molecules from the three *naked-pupa* mutants were unchanged by the same treatment (Fig. 2, lanes 1–4 and 5–8). These results suggest that the 30-kDa fhx/P25 contains terminal α1,2-mannose residues in their N-linked oligosaccharide chains, whereas such mannose residues are absent in the 27-kDa fhx/P25. The N-linked oligosaccharide chains were suggested to be retained in the 27-kDa fhx/P25 of J-139 because both 30- and 27-kDa forms were converted to the 24-kDa form by digestion with endo-H (Fig. 2, lane 9). The presence of N-linked oligosaccharide chains in the 27-kDa fhx/P25 molecules from the three *naked-pupa* mutants was shown previously by their positive reactions to biotinylated Con A and their susceptibility to the endo-H digestion [5].

The 27-kDa fhx/P25 is probably formed in Golgi complex

It was noted that fhx/P25 of the normal breed J-139, as detected with the anti-fhx/P25 antibody, was present as a single band (30 kDa) in sucrose-gradient fractions containing ER (Fig. 1A, fractions 8–16), whereas it was present as two bands (30 and 27 kDa) in fractions containing both ER and Golgi complex (Fig. 1A, fractions 1–6). In order to confirm these observations, fhx/P25 was partially purified from cocoon shells, ER extract, or ER plus Golgi mixed extracts of the normal breed C108 and subjected to Western blotting with the anti-fhx/P25 antibody or to the lectin blotting with biotinylated ConA before or after digestion with the bacterial α1,2-mannosidase or endo-H (Fig. 3A). Fhx/P25 was detected as a 30-kDa single band in the ER extract (Fig. 3A, lane 2) but as two bands (30 and 27 kDa) in the ER plus Golgi mixed extracts (Fig. 3A, lane 3) or in cocoons (Fig. 3A, lane 1). The 30-kDa fhx/P25 was converted to the 27-kDa single band by digestion with the bacterial α1,2-mannosidase (Fig. 3A, lanes 4–6), and the 30- and 27-kDa fhx/P25 were converted to the 24-kDa single band by digestion with endo-H (Fig. 3A, left panel, lanes 7–9). The reaction of fhx/P25 with biotinylated ConA became undetectable after digestion with endo-H (Fig. 3A, right panel, lanes 7–9). These results suggest that the 30-kDa component is the ER form of fhx/P25 and the 27-kDa component represents fhx/P25 whose N-linked oligosaccharide chains lost their terminal α1,2-mannose residues by digestion with α1,2-manosidases in Golgi complex, and further imply that fhx/P25 in the elementary unit of the normal-level fibroin-producing breeds is largely resistant to the action of α1,2-mannosidases in Golgi complex and secreted as the ER-type 30-kDa form.

Table 2. Determination of molar ratios of H-chain, L-chain, and fhx/P25 in the fibroin secreted into cocoons of a normal breed (C108), *Nd-s*^{D} mutant, or transgenic lines of *B. mori*. A_{490} values were obtained by ELISA. Protein samples (all in 100 μL) assayed were 10 ng for H-chain, 50 ng for L-chain and 125 ng for fhx/P25 from cocoons of C108 and the transgenic line L6 × 7, or 2 μg for H-chain, 10 μg for L-chain and 100 μg for fhx/P25 from *naked-pupa* cocoons of *Nd-s*^{D} *and* cocoons of the transgenic line L7-4. $n = 6$; ±, SD; ND, not detected.

Breed or line	A_{490}			ng/100 μg protein			pmol			Molar ratio		
	H-chain	L-chain	fhx/P25	H-chain	L-chain	fhx/P25	H-chain	L-chain	fhx/25	H-chain	: L-chain	: fhx/P25
C108	0.637	0.354	0.196	7.75×10^{4}	5.75×10^{3}	1.12×10^{3}	2.21×10^{4}	2.23×10^{4}	3.73×10^{3}	5.92 ± 0.04	: 5.98 ± 0.05	: 1
Nd-s^{D}	0.401	ND	0.421	245	ND	3	70	ND	11.1	6.31 ± 0.07	: –	: 1
Transgenic line												
L6×7	0.300	0.166	0.088	3.66×10^{4}	2.70×10^{3}	0.50×10^{3}	1.05×10^{4}	1.04×10^{4}	1.67×10^{3}	6.17 ± 0.05	: 6.16 ± 0.03	: 1
L7-4	0.625	0.344	0.186	761	5.59	1.06	218	215	35.3	6.18 ± 0.03	: 6.09 ± 0.04	: 1

A role of L-chain in the protection of α1,2-mannose residues of the N-linked oligosaccharide chains of fhx/P25 in the elementary unit

The PSG cell extract of *Nd-s*^{D} mutant was subjected to the sucrose density gradient centrifugation to separate ER and ER plus Golgi mixed fractions as for the normal breed J-139 (Fig. 1B), and each fraction was sonicated briefly to yield an extract. When electrophoretic mobilities of partially purified fhx/P25 molecules from cocoon shells, the ER extract and the ER plus Golgi mixed extracts from *Nd-s*^{D} mutant were compared, it was confirmed that only the 27-kDa fhx/P25 was present in the extremely small amount of fibroin secreted into cocoons (Fig. 3B, lane 1), whereas only the 30-kDa form was present in the ER extract and both 30- (major) and 27-kDa (minor) forms were present in the ER plus Golgi mixed extracts (Fig. 3B, lanes 2, 3) as for the normal breed C108 (Fig. 3A). It is thus conceivable that in the *Nd-s*^{D} mutant silkworm, fhx/P25 in the L-chain-free H_6fhx_1-type elementary unit (Table 2) is processed efficiently by the action of Golgi α1,2-mannosidases to yield only the 27-kDa molecule in the secreted fibroin.

In order to examine a role of L-chain in the protection of α1,2-mannose residues of fhx/P25 in the elementary unit, the *Nd-s*^{D} mutant silkworm was subjected to transgenesis with the normal L-chain promoter/cDNA sequence together with a marker gene of DsRed2 (Fig. 4A), and two transgenic lines L6 × 7 and L7-4 were selected. The L6 × 7 line expressed DsRed2 strongly and the L-chain sequence was suggested to be integrated into two major genomic loci by Southern blot hybridization (Fig. 4B, lanes 2 and 3) with a probe specific to the normal L-chain gene (Fig. 4A). Northern blot hybridization indicated that both normal L-chain mRNA and *Nd-s*^{D} mutant L-chain mRNA were produced at high levels in a PSG-specific manner in the L6 × 7 line (Fig. 4C, lanes 2 and 3). Another transgenic line L7-4 expressed extremely low-levels of DsRed2 and the normal L-chain mRNA as judged by reverse transcription (RT)-PCR (data not shown). In consistency with these results, the mean weight of cocoons produced ($n = 50$ each) was 110 mg for L6 × 7 and 20.1 mg for L7-4 as compared with 187 mg for C108 and 17.7 mg (consisting mostly of sericin) for *Nd-s*^{D}.

Proteins from cocoon shells of C108 (2.5 μg; Fig. 5, lanes 1 and 5), *Nd-s*^{D} (200 μg; Fig. 5, lanes 2 and 6) and two transgenic lines [L6 × 7 (5 μg; Fig. 5, lanes 3 and 7) and L7-4 (200 μg; Fig. 5, lanes 4 and 8)] were subjected to Western blotting with the anti-H-chain (Fig. 5A), the anti-L-chain (Fig. 5B), or the anti-fhx/P25 Ig (Fig. 5C), and to the lectin blotting using biotinylated ConA (Fig. 5D) before (Fig. 5, lanes 1–4) or after (Fig. 5, lanes 5–8) the reductive cleavage of disulfide bonds. In these experiments, a large excess of cocoon protein samples was used from *Nd-s*^{D} and the transgenic line L7-4 in order to obtain similar signal intensity. In all the cocoon protein samples, H-chain (Fig. 5A) and fhx/P25 (Fig. 5C) were detected. The normal L-chain was detected clearly after cleavage of disulfide bonds (Fig. 5B) in the normal (C108; lane 5) and the two transgenic lines (lanes 7 and 8) but the mutant L-chain was undetectable in the cocoon protein sample from the *Nd-s*^{D} mutant (Fig. 5B, lanes 2 and 6). The anti-L-chain polyclonal antibody [1] used in these experiments could detect the *Nd-s*^{D} mutant L-chain as well in the PSG cell extract (Fig. 1B). It was suggested from these results that the relatively large amount of normal L-chain expressed in the L6 × 7 transgenic line contributed to the restoration of the high-level secretion of fibroin.

In the two transgenic lines (L6 × 7 and L7-4), fhx/P25 was detected as two bands of 30 and 27 kDa (Fig. 5C, lanes 3, 7 and 4, 8) like in the normal breed C108 (Fig. 5C, lanes 1 and 5). The slight decrease in electrophoretic mobility of fhx/P25 after cleavage of disulfide bonds was interpreted as the loss of the compact structure of fhx/P25 due to the cleavage of intramolecular disulfide bonds [5]. In the transgenic lines, fhx/P25 molecules of 30 and 27 kDa were both reactive to biotinylated ConA indicating that N-linked oligosaccharide chains existed in both types of molecules (Fig. 5D). It was demonstrated by the quantitative ELISA that the molar ratios of H-chain, L-chain, and fhx/P25 were close to 6 : 6 : 1 for the fibroin secreted into cocoons of both transgenic lines (Table 2).

Discussion

Biological significance of the assembly of the elementary unit of fibroin in ER

It was demonstrated in this study that the elementary unit of fibroin, having the same molar ratio of H- and L-chains, and fhx/P25 as of the elementary unit present in the PSG extract, could be isolated from the ER extract of PSG cells of a normal-level fibroin-producing silkworm

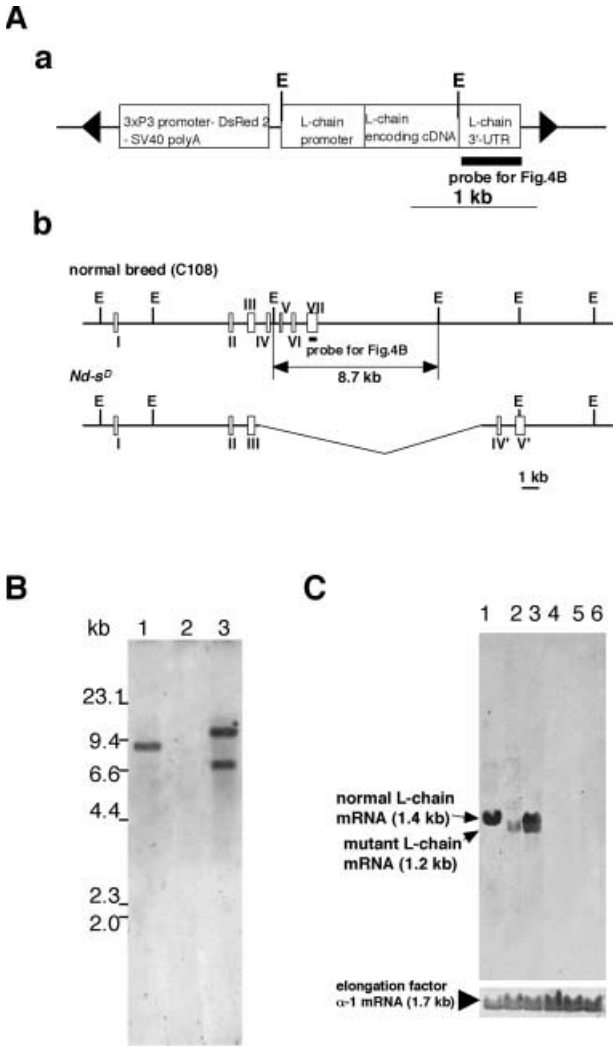

Fig. 4. Characterization of the transgenic silkworm line L6 × 7. (A) Illustration of a recombinant transforming vector and normal and mutant *L-chain* genes. (a) Arrangement of two genes in pBac(3xP3-DsRed2 + L-chain promoter/normal L-chain cDNA) recombinant vector used for the transgenesis of *Nd-s*^{D} mutant silkworm. The *DsRed2* marker gene construct is placed immediately downstream of the Pax-6 promoter sequences (3xP3) enabling its expression in the photoreceptor cells. Inverted terminal repeats (arrows) and *Eco*RI sites (E) are indicated. (b) Physical maps of the normal (C108) and the *Nd-s*^{D} mutant *L-chain* genes. The normal gene (*fib-L*) contains seven exons (I to VII), whereas the chimeric mutant gene contains exons I to III of *fib-L* and exons IV′ and V′ derived from the far downstream region. (B) Southern blot hybridization of *Eco*RI-digested genomic DNAs (5 μg per lane) from C108 (lane 1), *Nd-s*^{D} (lane 2) and L6 × 7 (lane 3) with the normal L-chain gene-specific probe as shown in A (a) and (b). (C) Northern blot hybridization of total RNAs (5 μg per lane) from PSG (lanes 1–3) or MSG (lanes 4–6) of C108 (lanes 1 and 4), *Nd-s*^{D} (lanes 2 and 5) or L6 × 7 (lanes 3 and 6) with the 357-bp L-chain cDNA probe (encoded by exons I, II, and III) common to C108 and *Nd-s*^{D} L-chain mRNAs or with the cDNA probe for the *B. mori* elongation factor α-1 isoform as an internal control.

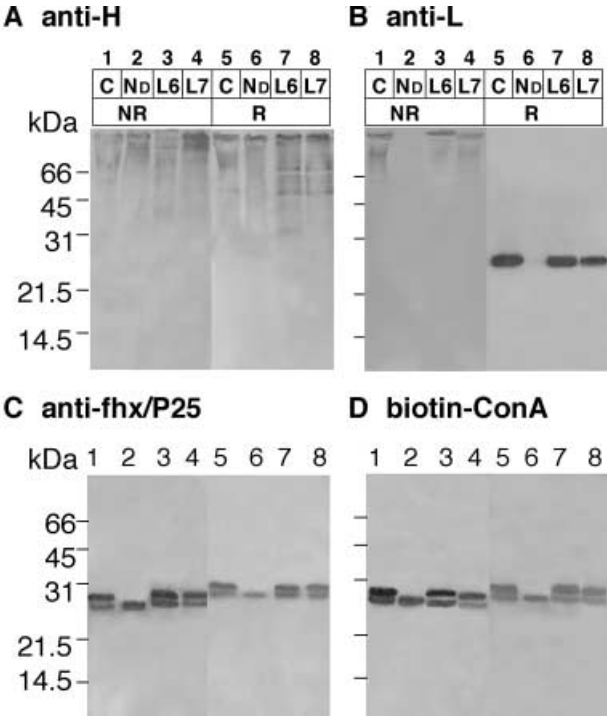

Fig. 5. Analysis of H-chain, L-chain and two different forms of fhx/P25 in protein samples from cocoon shells of the normal breed, *Nd-s*^{D} mutant and two transgenic lines. Proteins from cocoon shells of a normal breed C108 (C; lanes 1 and 5), *Nd-s*^{D} (ND; lanes 2 and 6), L6 × 7 (L6; lanes 3 and 7), or L7-4 (L7; lanes 4 and 8) were separated by SDS/PAGE before (NR; lanes 1–4) or after (R; lanes 5–8) the reductive cleavage of disulfide bonds and subjected to Western blotting with the anti-H-chain Ig (A), the anti-L-chain Ig (common to normal and *Nd-s*^{D} L-chains) (B), the anti-fhx/P25 Ig (C), or to the lectin blotting with biotinylated ConA (D).

breed J-139. It has been shown that the assembly of oligomeric protein complexes occurs generally in ER [24] but some protein complexes, such as connexin 43 of gap junctions [25] and M protein of coronavirus [26], have been shown to be assembled in Golgi complex. In the case of silk fibroin, a vast amount of newly synthesized, high molecular-mass (≈ 350 kDa) fibrous H-chain molecules in ER must be transported efficiently, without being denatured, to Golgi complex for secretion from PSG cells. The assembly of the elementary unit in ER is most likely a crucial event to meet the ER quality control [27] and to ensure the efficient intracellular transport and secretion of H-chains.

There seem to be two key events in the assembly of the $(H\text{-}L)_6fhx_1$-type elementary unit: (a) the formation of disulfide-linked H-L heterodimers and (b) the formation of the three-component protein complex in which the non-covalent association of the glycoprotein fhx/P25 is centrally important. The clue to the first event has been obtained from studies [8] on the *naked-pupa* mutants, *Nd-s* and *Nd-s*^{D}. In these mutant silkworms, chimeric L-chains are formed because of the exon-shuffling mutation of the *L-chain* gene. These chimeric L-chains lack Cys172 which normally forms a disulfide bond with Cys-c20 (the 20th residue from the C-terminus) of the H-chain. The free sulfhydryl group of Cys-c20 on H-chains seem to be harmful for the transport of the elementary unit from ER to Golgi, because (a) the level of fibroin secretion is less than

1% of the normal level, (b) ER is unusually enlarged in PSG cells, (c) the development of PSG is significantly retarded and (d) a thin, *naked-pupa*-type cocoons are formed in these mutants [7]. The present results that (a) the normal L-chain is expressed (b) the normal elementary unit is assembled and (c) the *Nd-s*^{D} phenotypes with respect to the retardation of PSG development and the formation of thin cocoons were largely converted to the normal phenotypes in the L6 × 7 transgenic line expressing a significant level of the normal L-chain strongly support the above notion.

With respect to the second event in the assembly of the elementary unit, it is of interest to note that the extremely small amount of fibroin secreted into thin cocoons of the *Nd-s*^{D} mutant formed the H_6fhx_1-type complex (Table 2). These results suggest that, although very inefficient in the absence of L-chain, one molecule of fhx/P25 could associate noncovalently with six molecules of H-chains. We demonstrated previously that N-linked oligosaccharide chains of fhx/P25 were important in maintaining the integrity of the elementary unit [1]. However, considering the facts that oligosaccharide chains play important roles in the assembly of vesicular stomatitis viral glycoprotein [28,29], influenza virus hemagglutinin [30,31], and IgM [32] in ER, a further role of N-linked oligosacharide chains of fhx/P25 in the process of assembly of the elementary unit in ER may be speculated. With regard to this notion, the present finding that a relatively large amount of fhx/P25 molecules, i.e. about one third of molecular numbers of H-chain and L-chain (Table 1), exists in ER without being assembled into the elementary unit is interesting. This observation seems to be consistent with the previous observation that concentrations of fhx/P25 mRNA and H-chain mRNA are roughly equimolar in PSG cells during the last intermolt stage [33]. The deduced sequence of fhx/P25 contains a typical signal peptide sequence at the N-terminal region [34] and the sequence is cleaved at the N-terminal side of Gly18 in the secreted fhx/P25 [1]. However, fhx/P25 does not contain the ER retention signal sequence [35] in its C-terminal region. Thus, it could be speculated that the retention of fhx/P25 molecules which were not assembled into the elementary unit in ER might be attained by interaction with other ER-resident proteins but the real reason remains to be elucidated.

We would like to speculate that fhx/P25 molecules having N-linked oligosaccharide chains interact noncovalently with nascent H-chains, perhaps during their translation and translocation into ER, as a sort of molecular chaperone to prevent denaturation of H-chains. Subsequently L-chains form a disulfide bond with Cys-c20 of H-chains, then assembly of the $(H\text{-}L)_6fhx_1$ complex takes place. The successfully assembled elementary units are allowed by the ER quality control system and transported efficiently to Golgi complex.

Two types of N-linked oligosaccharide chains of fhx/P25

It has been shown that most of the glycoproteins expressed in lepidopteran insect cells have either high-mannose type ($GlcNAc_2Man_{5-9}$) or truncated tri-mannosyl type [$GlcNAc_2Man_3(\pm$ Fuc)] N-linked oligosaccharide chains, most likely due to the high activity of β-*N*-acetylglucosaminidase in Golgi, which prevents further processing to the complex- or hybrid-type chains [36–38]. As suggested from the deduced primary structure of fhx/P25 [2,3], the presence of three N-linked oligosaccharide chains per a molecule of fhx/P25 was demonstrated [1]. Furthermore, conversion of the 30-kDa form of the purified or partially purified fhx/P25 to the 27-kDa form was achieved by digestion with the bacterial α1,2-mannosidase (this study). We can calculate molecular masses of fhx/P25 containing different numbers of mannose residues as follows: 29.74 kDa for a molecule containing three chains of $GlcNAc_2Man_9$ and 27.58 kDa for a molecule containing three chains of $GlcNAc_2Man_5$, which are close to the 30- and 27-kDa forms of fhx/P25, respectively.

We think it most probable that the 27-kDa form of fhx/P25 is produced in the Golgi complex by the action of Golgi α1,2-mannosidases, because the present results showed that only the 30-kDa form was present in the ER extract and the 27-kDa form was detected in the Golgi plus ER mixed extracts from PSG cells. In the giant polyploid cells of PSG, ER is fully developed [39] and we could not succeed in isolating a pure Golgi fraction which is not contaminated with ER. We also tried to compare digestibility of the terminal α1,2-mannose residues of N-linked oligosaccharide chains of fhx/P25 in the isolated intact elementary unit or in the L-chain-free elementary unit produced by the treatment with dithiothreitol as described [1] with the bacterial α1,2-mannosidase *in vitro*. However, this enzyme could not cleave the α1,2-mannose residues of fhx/P25 in either protein complex (data not shown), although the same enzyme could digest α1,2-mannose residues efficiently in purified or partially purified fhx/P25. We suspect that the dynamic configuration of the isolated elementary unit is different from that during the intracellular transport or the size of the bacterial α1,2-mannosidase dimer (180 kDa × 2 = 360 kDa) employed [17] is too large to access the oligosaccharide chains of fhx/P25 which have been suggested to be located internally of the elementary unit [1].

A possible mechanism of the protection of α1,2-mannose residues of fhx/P25 in the elementary unit in the presence of L-chain

Two possibilities exist with respect to the role of L-chain in the protection of terminal α1,2-mannose residues of fhx/P25 in the elementary unit from the action of Golgi α1,2-mannosidases: a direct model (Fig. 6A) and an indirect model (Fig. 6B). In the direct model, the L-chain has dual functions: formation of the disulfide-linked H–L heterodimer which is essential for the efficient transport of the H-chain-containing protein complex from ER to Golgi and protection of the terminal α1,2-mannose residues of fhx/P25 in the elementary unit. We suggested previously that the six sets of H–L dimers surrounded the centrally located fhx/P25 molecule in the elementary unit based on the observation that biotinylated ConA was accessible to the N-linked oligosaccharide chains of fhx/P25 only after the treatment of the elementary unit with 2 M urea or 2% Triton X-100 [1]. The L-chains in the majority of elementary units may interact noncovalently with the oligosaccharide chains of fhx/P25 or act as structural barriers and cause protection of the terminal mannose residues from digestion with α1,2-mannosidases in Golgi complex.

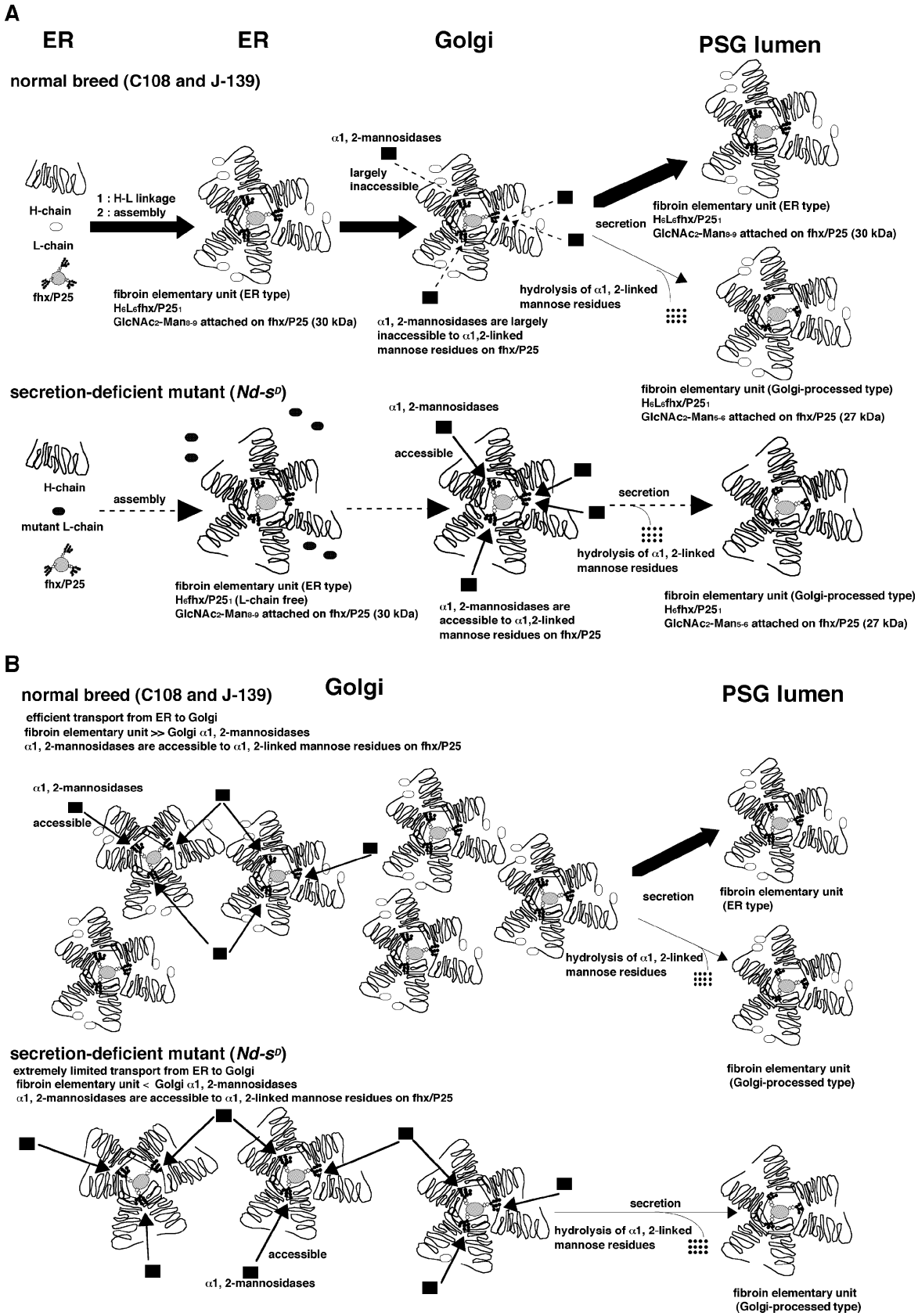

Fig. 6. Models for a role of L-chain in the protection of α1,2-mannose residues in N-linked oligosaccharide chains of fhx/P25 in the elementary unit of fibroin from digestion with Golgi α1,2-mannosidases. (A) A direct model and (B) an indirect model.

In the latter model, the function of the L-chain is to accelerate transport of the assembled elementary unit from ER to Golgi. A vast flow of elementary units containing H–L dimers and fhx/P25 into Golgi may result in conditions where molecular numbers of Golgi α1,2-mannosidases become substantially insufficient and thus the majority of elementary units are allowed to leave Golgi for secretion without losing the α1,2-mannose residues of fhx/P25.

It is of interest to note that 30- and 27-kDa forms of fhx/P25 are present in either transgenic line; L6 × 7 producing a high level or L7-4 producing an extremely low level of normal L-chain. It implies that numbers of the normal elementary unit are assembled in proportion to the available numbers of the normal L-chain, and the N-linked oligosaccharide chains of fhx/P25 in those elementary units are protected to the similar extent from the action of Golgi α1,2-mannosidases. These results seem to favor the direct model. On the other hand, the presence of 27-kDa fhx/P25 as a minor component in elementary units produced in the normal breeds seems to favor the indirect model. Perhaps the above two models may not be mutually exclusive. Although further experimental evidences are required to judge which mechanism is more likely, present results suggest that not only fhx/P25 but also L-chain play respective roles in the formation of the integral structure of the elementary unit of fibroin.

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