

Monoclonal Antibodies to the Light-harvesting Chlorophyll *a/b* Protein Complex of Photosystem II

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Abstract. A collection of 17 monoclonal antibodies elicited against the light-harvesting chlorophyll *a/b* protein complex which serves photosystem II (LHC-II) of *Pisum sativum* shows six classes of binding specificity. Antibodies of two of the classes recognize a single polypeptide (the 28- or the 26-kD polypeptides), thereby suggesting that the two proteins are not derived from a common precursor. Other classes of antibodies cross-react with several polypeptides of LHC-II or with polypeptides of both LHC-II and the light-harvesting chlorophyll *a/b* polypeptides of photosystem I (LHC-I), indicating that there are structural similarities among the polypeptides of LHC-II and LHC-I. The evidence for protein processing by which the 26-, 25.5-, and 24.5-kD polypeptides are derived

from a common precursor polypeptide is discussed.

Binding studies using antibodies specific for individual LHC-II polypeptides were used to quantify the number of antigenic polypeptides in the thylakoid membrane. 27 copies of the 26-kD polypeptide and two copies of the 28-kD polypeptide were found per 400 chlorophylls. In the *chlorina f2* mutant of barley, and in intermittent light-treated barley seedlings, the amount of the 26-kD polypeptide in the thylakoid membranes was greatly reduced, while the amount of 28-kD polypeptide was apparently not affected. We propose that stable insertion and assembly of the 28-kD polypeptide, unlike the 26-kD polypeptide, is not regulated by the presence of chlorophyll *b*.

THE light-harvesting chlorophyll *a/b* pigment-protein complex (LHC-II),¹ which preferentially sensitizes photosystem II (PSII), functions by absorbing light energy and transferring it to the reaction center core complex. Much research has been done on the chromophore and polypeptide composition of LHC-II, the genes encoding the major polypeptide, the developmental regulation of LHC-II, and the function of the complex in excitation energy distribution (5, 17, 38). LHC-II contains several polypeptides ranging from 23 to 29 kD (apparent molecular mass by SDS PAGE) in size (31) that are similar in amino acid composition (1, 19, 35). However, little progress has been made in identifying the function of individual polypeptides. This problem has been complicated by the recent discovery that at least one size class of the LHC-II polypeptides is encoded by a large nuclear gene family (9, 13, 40). In the case where the DNA sequences of individual genes representing several subfamilies have been examined, low levels of sequence diversity have been identified (12). It is not known if the resulting difference in amino acid composition is enough to modify the function of the polypeptides. Thus, it is possible that the LHC-II polypeptides expressed in the membrane are some-

what diverse, making the assembled LHC-II heterogeneous both in the size and in the primary structure of its polypeptides.

To correlate the presence of a specific LHC-II polypeptide in thylakoids with functional parameters, it is necessary to have an analytical tool that can be used to identify the individual proteins. To this end, we have prepared a collection of monoclonal antibodies to the isolated complex from pea thylakoid membranes. Monoclonal antibodies have the potential to discriminate between antigens of different LHC-II polypeptides on the basis of small variations in structure. Each antibody preparation contains only a single species of immunoglobulin molecule that will bind a single, specific, antigenic site in the LHC-II (21). Thus the observation of cross-reactivity or lack of it between two polypeptides identifies the presence or absence of common structural regions. This paper characterizes our initial collection of monoclonal antibodies and describes the use of two of them to study the 28- and the 26-kD polypeptides of LHC-II.

Materials and Methods

Isolation of LHC-II

Pisum sativum var. Progress No. 9 (Ferry Morse Seed Co., Mountain View, CA) was grown in growth chambers under a 16-h day, 8-h night photoperiod and ambient humidity. Day and night temperatures were maintained at 20°

1. *Abbreviations used in this paper:* IML, intermittent light; LHC-I, light-harvesting chlorophyll *a/b* protein complex serving photosystem I; LHC-II, light-harvesting chlorophyll *a/b* protein complex serving photosystem II; PSI, photosystem I; PSII, photosystem II.

and 18°C, respectively. Seedlings were used for preparation of LHC-II ~14 d after germination.

LHC-II was isolated using the method of Burke et al. (8), with some modification. 100 g of shoot tissue was homogenized in 300 ml of 0.1 M Tricine-NaOH, pH 7.8, and 0.4 M sorbitol at 4°C. The slurry was filtered through 12 layers of cheesecloth to remove cell fragments, and the filtrate was centrifuged at 1,000 g for 10 min. The pellet was resuspended in 200 ml of a solution of 0.1 M sorbitol, 0.75 mM EDTA, pH 7.8, and centrifuged at 10,000 g for 5 min. The pellet was washed once more in ~100 ml of a 0.1 M sorbitol solution and centrifuged at 10,000 g for 5 min. This final pellet was resuspended in distilled water and the chlorophyll concentration was measured by the method of MacKinney (29). The membranes were diluted with distilled water to a final concentration of 0.8 mg chlorophyll/ml, and a tissue homogenizer was used to thoroughly disperse clumps of thylakoids. Sufficient Triton X-100 (Sigma Chemical Co., St. Louis, MO) was added with stirring to yield a final concentration of 0.78% (wt/vol). This mixture was stirred in the dark for 30 min at 25°C. Unsolubilized membranes were removed by centrifugation at 43,000 g for 30 min. The solubilized material was loaded onto 0.1–1.0 M linear sucrose density gradients containing 0.02% Triton X-100 (wt/vol), and centrifuged in a swinging bucket rotor at 100,000 g for 16 h. During centrifugation a broad green band formed over the top one-third of the gradient. When this region of the tube was illuminated from the side with a high intensity lamp, a band exhibiting high chlorophyll fluorescence was clearly visible. This band was collected and LHC-II was purified from the solution by aggregation with Mg²⁺ ions. The solution was made 5 mM MgCl₂ and stirred at room temperature for 10–15 min. Aggregated protein was separated by centrifugation at 18,000 g for 15 min and the pellet was resuspended in 10 mM Tricine-NaOH, pH 7.8, 10 mM NaCl.

Preparation of Monoclonal Antibodies

Three 8-wk-old female BALB/c mice were injected with 100 µg of purified LHC-II protein subcutaneously in 200 µl of a 50% (vol/vol) complete Freund's adjuvant solution. 27 d later they were injected intraperitoneally (i.p.) with 100 µg of LHC-II in 150 µl phosphate-buffered saline (PBS; 10 mM NaH₂PO₄, pH 7.3, 150 mM NaCl). 4 d later 50-µl samples of serum were collected from each mouse and tested for the presence of antibodies specific for LHC-II using an enzyme-linked immunosorbent assay (ELISA). All three mice exhibited a good immune response to the antigen. The i.p. injection was repeated on days 42 and 53. Spleen cells were harvested 3 d after the final injection. The cells were fused to mouse myeloma Sp2/0-Ag14 cells in the ratio of 5:1 using 35% (wt/vol) polyethylene glycol 1000 and 5% (vol/vol) dimethylsulfoxide (14). 12 d after fusion, the hybridoma cultures were screened for the production of antibodies specific to isolated LHC-II using ELISA. Of 1,522 initial colonies, 13 were selected and cloned by limiting dilution. Approximately 100 clones of each original colony were tested in ELISA for production of antibodies specific to LHC-II. Six clones of each set of 100 were selected and screened for specificity to LHC-II polypeptides using Western blot analysis. 14 clones were finally chosen. They were divided into six classes according to their binding specificity for LHC-II polypeptides in Western blots. At least one member of each class was cloned a second time by limiting dilution to verify that it was truly monoclonal. Western blot analysis of six of the resulting clones from each line showed no variation in the polypeptide binding pattern.

In a separate set of experiments, five hybridoma lines secreting antibodies specific for LHC-II were identified in a collection of hybridoma colonies prepared from mice injected with barley leaf cell membrane fractions. These hybridoma lines were prepared and cloned twice as described above.

Antibody subclasses were identified using a mouse monoclonal subtyping kit (HyClone Laboratories, Logan, UT).

ELISA

ELISA was done using a streptavidin HyBRL Screen Kit (Bethesda Research Laboratories, Gaithersburg, MD). A Nunc 96-microwell Immuno Plate I F (Gibco, Grand Island, NY) was coated with 0.75 µg of LHC-II protein in 50 µl of 50 mM Na-carbonate, pH 9.2, per well by incubation at 4°C for 12 h. The rest of the assay was carried out following the kit's instructions.

Western Blots

PAGE was done using the method of Laemmli (24). A gradient of acrylamide from 11 to 17% (wt/vol) was used to increase resolution. Protein samples in sample buffer (2% [wt/vol] SDS, 2% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 0.0625 M Tris-HCl pH 6.8, and 0.01% [wt/vol]

bromophenol blue) were boiled for 3 min before loading on the gel to thoroughly dissociate the chlorophyll from the protein. 8 µg of thylakoid chlorophyll or 1.5 µg of LHC-II chlorophyll was loaded in a 1-cm-wide lane. A portion of each gel was cut and stained with Coomassie Blue (0.1% [wt/vol] Coomassie Brilliant Blue R-250, 7% [vol/vol] acetic acid, 50% [vol/vol] methanol), and destained in a solution of 7% (vol/vol) acetic acid, 20% (vol/vol) methanol, and 3% (vol/vol) glycerol.

Western blots were prepared using the method of Towbin et al. (41). The gel was sandwiched next to a sheet of nitrocellulose (Schleicher & Schuell, Keene, NH) and immersed in 192 mM glycine, 25 mM Tris-HCl, pH 8.3, 20% (vol/vol) methanol. The protein was subjected to electrophoresis out of the gel and onto the surface of the nitrocellulose filter (60 V, 6 h, at room temperature). After electrophoresis all remaining protein binding sites on the nitrocellulose were blocked by soaking the sheet in a solution of 1% BSA (wt/vol), 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, for 6 h. The Western blot development procedure was modified from the method of Tsang et al. (42). The monoclonal antibody solution (spent culture fluid, or partially purified ascites solution) was incubated with the nitrocellulose in TST solution (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% [vol/vol] Tween-20), containing 1% BSA for 2 h at 37°C. The blot was then washed for 15 min in TST without BSA four times at 25°C with continuous shaking. Alkaline phosphate-conjugated goat anti-mouse immunoglobulin (Cooper Biomedical Inc., Malvern, PA) was diluted 1:500 in TST solution and incubated with the nitrocellulose for 2 h at 25°C. The blots were washed and developed using the method of Leary et al. (26). Unbound conjugated antibody was removed by washing the blots for 15 min with 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.3% (vol/vol) Tween-20 (AP 7.5 solution) three times and for 15 min with 100 mM Tris-NaOH, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ (AP 9.5 solution) two times. For color development the blots were incubated with a solution of 0.33 mg/ml nitro blue tetrazolium, 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) in the AP 9.5 solution. The bromochloroindolyl phosphate was first dissolved in dimethylformamide (0.05 mg/µl dimethylformamide) before it was added to the nitro blue tetrazolium solution. The color reaction was allowed to proceed 10–15 min in the dark. The reaction was stopped by washing the blots first for 10–15 min in a solution of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and then for 10–15 min in a solution of 20 mM Tris-HCl, pH 9.5, 5 mM EDTA for 10–15 min. The blots were dried between sheets of blotting paper and photographed.

Ascites Production

To produce large quantities of monoclonal antibodies, ascites tumors were induced. 14 8-wk-old mice were injected i.p. with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, WI). This injection was repeated 7 d later. On day 14, the mice were injected i.p. with 3 × 10⁶ hybridoma cells in 0.3 ml of serum-free culture media (7). Seven mice were used for each cell line. Ascites tumors developed in ~1 wk and the fluid was collected on alternate days for 8–12 d. Fluid produced by mice injected with the same cell line was pooled and frozen at -20°C until further use.

Upon thawing, the fluid was centrifuged at 100,000 g for 30 min to remove aggregated material. The supernatant volume was measured and then diluted 1:1 with cold PBS. Cold saturated (NH₄)₂SO₄ was added with stirring to yield 50% saturation, and the mixture was stirred on ice for 45 min. Precipitated protein was collected by centrifugation at 10,000 g for 30 min. The pellet was resuspended with a small volume of a solution of 20 mM Tris-HCl, pH 7.4, 10 mM NaCl and dialyzed against three changes of the same buffer for 24 h. The dialyzed solution was aliquoted into single-use vials and stored at -20°C. The concentration of antibody was measured using a Serotec radial immunodiffusion plate and Serotec antibody standard solutions (Serotec, Bicester, England).

The antibodies were labeled in vitro using Amersham ³⁵S-labeling reagent according to the manufacturer's instructions (SLR, 1 mCi/ml, Amersham Corp., Arlington Heights, IL).

Binding Assays

Unstacked thylakoid membranes were prepared from *Pisum sativum* var. Progress No. 9 by homogenizing 50 g of shoot tissue in a chilled solution of 50 mM Tricine-NaOH, pH 7.8, 400 mM sorbitol, 10 mM NaCl. The slurry was filtered through 12 layers of cheesecloth and centrifuged at 1,500 g for 10 min. The pellet was resuspended in a low osmotic solution of 10 mM Tricine-NaOH, pH 7.8, and 10 mM NaCl to break the chloroplast envelope membrane. The thylakoid membranes were pelleted from this solution by centrifugation at 10,000 g for 5 min. The final pellet was

resuspended in 10 mM Tricine-NaOH pH 7.8, 10 mM NaCl, 100 mM sorbitol and held on ice until use in the binding assay. Chlorophyll concentrations were measured by the method of MacKinney (29).

For the binding assay, unstacked thylakoid membranes were diluted into PBS containing 100 mM sorbitol and 0.25% (wt/vol) BSA. The solution was divided into 0.5-ml aliquots in 1.5-ml Eppendorf tubes and labeled antibody was added immediately. The antibody was incubated with the membranes for 2 h at 37°C on a rotary shaker. After this incubation, the membranes were pelleted by centrifugation for 2 min in a microfuge and the supernatant was removed by aspiration. The membranes were washed three times by resuspension in the PBS/sorbitol/BSA solution and centrifugation. The final pellet was resuspended in 100 µl of PBS, added to 5 ml of scintillation cocktail, and the radioactivity measured in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA).

Isolation of Photosystem I (PSI)

PSI was prepared from *Pisum sativum* var. Progress No. 9 thylakoid membranes using the method of Mullet et al. (32). For Western blots, PSI samples were solubilized and boiled as described above; 7.5 µg of chlorophyll was loaded per 1-cm lane.

Intermittent Light (IML) Treatment

Hordeum vulgare var. Morex (Michigan Seed Foundation, East Lansing, MI) was grown in the dark for 7 d and then exposed to IML (2 min light, 2 h dark cycles) for 48 h. Seedlings to be used as controls were grown in a growth chamber under a 16 h photoperiod at 21°C day and 18°C night temperatures. Chlorophyll *b* deficient barley mutants (*chlorina f2*) were grown under similar conditions. Thylakoids were isolated as previously described (11).

Trypsin Treatment

Isolated LHC-II was proteolytically digested by incubating LHC-II (300 µg chlorophyll/ml) with trypsin (1 µg/ml; bovine pancreas type III, Sigma Chemical Co.) in 100 mM Tricine-NaOH, pH 7.8, 100 mM sorbitol, 10 mM NaCl, 5 mM MgCl₂ at 25°C. The reaction was stopped at various time points by adding an aliquot of the solution to the gel electrophoresis sample buffer and heating it to 100°C.

For the binding assays, pea thylakoid membranes (100 µg chlorophyll/ml) were incubated with trypsin (0.25 µg/ml) at 25°C for 20 min as above. The thylakoids were then washed three times in the PBS/sorbitol/BSA solution before use in the binding assay.

Results

Characterization of the Antibody Collection

19 hybridoma cell lines producing monoclonal antibodies specific for LHC-II have been identified and cloned. Antibody binding to chloroplast thylakoid proteins has been characterized by Western blot analysis. On the basis of their polypeptide recognition, 14 of the antibodies have been divided into six classes which are summarized in Table I. Five additional monoclonal antibodies failed to bind LHC-II

Table I. Summary of Binding Specificity of the Monoclonal Antibody Collection

Class	No. of members	LHC-II				LHC-I		
		28	26	25.5	24.5	24	23.5	21.5
I	1	—	X	—	—	—	—	—
II	1	X	—	—	—	—	—	—
III	3	—	X	X	X	—	—	—
IV	4	X	X	X	X	—	—	—
V	1	—	X	X	X	—	X	X
VI	4	X	X	X	X	X	X	X

X, binding of antibody to polypeptide. —, no binding.

polypeptides in Western blots even though they produced strong reactions with the Triton-solubilized LHC-II in ELISA. We assumed that these antibodies bind to conformationally determined epitopes on the LHC-II. They were not further characterized.

LHC-II antibodies that reacted on Western blots showed two types of reactions: recognition of a single polypeptide or reaction with multiple polypeptides. Of the latter type of reaction, there was a further distinction obvious in Western blots of intact thylakoids; certain monoclonal antibodies reacted with only LHC-II polypeptides, whereas others reacted with both LHC-II and the light-harvesting chlorophyll *a/b* proteins that are exclusively associated with the PSI core complex (LHC-I) (16, 23, 25, 32).

Fig. 1 shows an example of two single-protein specific antibodies, designated class I and II, which react with the 26- and 28-kD proteins of the thylakoid, respectively. To verify that the antibodies were reacting with authentic LHC-II polypeptides, Western blots of purified LHC-II preparations are shown in Fig. 2. The Coomassie-stained lanes (Fig. 1, lane *d* and Fig. 2, lane *a*) showed four polypeptides in the LHC-II preparation, with apparent molecular masses of 28, 26, 25.5, and 24.5 kD. The monoclonal antibody of class I (antibody MLH1) reacted strongly with the dominant 26-kD polypeptide component of the purified LHC-II preparation (Fig. 1, lane *b*, and Fig. 2, lane *b*). In contrast, the class II antibody (antibody MLH2) reacted solely with the minor 28-kD polypeptide (Fig. 1, lane *a*, and Fig. 2, lane *d*). These two proteins can be clearly resolved in the Western blot analysis using a mixture of antibodies MLH1 and MLH2 (Fig. 2, lane *c*). As a control, a Western blot with polyclonal mouse antibodies elicited against the LHC-II complex are

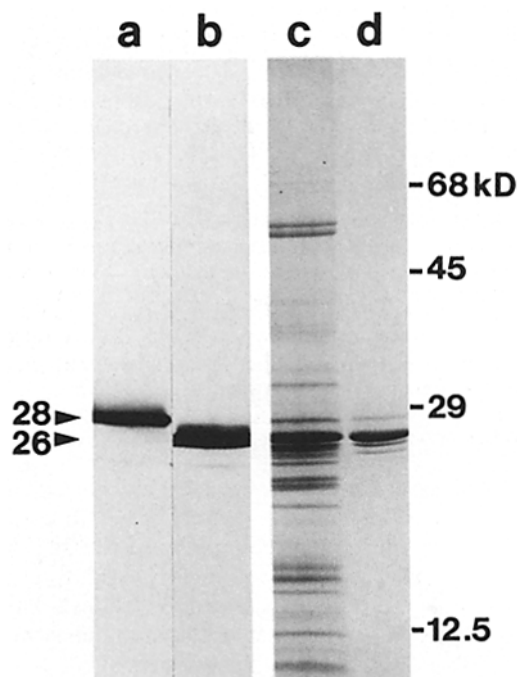


Figure 1. Western blot and stained PAGE of pea thylakoid membranes probed with monoclonal antibodies MLH1 and MLH2. Lanes *a* and *b*, Western blot of thylakoid membranes probed with MLH2 (*a*) and MLH1 (*b*). Lanes *c* and *d*, Coomassie Blue-stained PAGE of thylakoid membranes (*c*) and purified LHC-II (*d*).

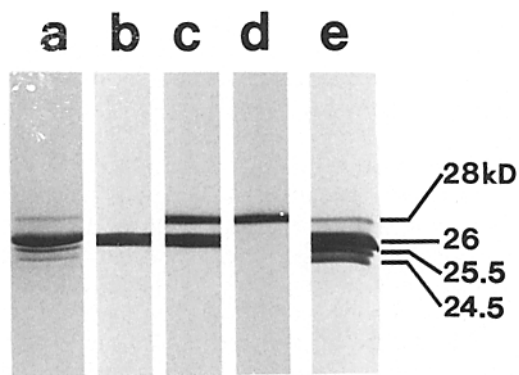


Figure 2. Western blot and stained PAGE of LHC-II isolated from pea thylakoid membranes. Lane *a*, stained PAGE of isolated LHC-II. Lanes *b-e*, Western blot of LHC-II probed with MLH1 (*b*); a mixture of MLH1 and MLH2 (*c*); MLH2 (*d*); polyclonal LHC-II antibodies (*e*).

shown in Fig. 2, lane *e* to demonstrate that all four stained LHC-II polypeptides are present on the nitrocellulose filter. MLH1 and MLH2 bind polypeptides equivalent to the 26- and 28-kD polypeptides in other plant species such as barley, maize, and tomato (data not presented).

Western blots of both purified pea LHC-II and pea thylakoid membranes using antibodies from class III (antibody MLH5) and class IV (antibody MLH8) are shown in Fig. 3. Two members of class IV also recognize an unidentified polypeptide of ~ 20 kD which is present in thylakoid membranes (data not shown). This polypeptide is not present in PSI preparations. It may be a breakdown product of LHC-II, or perhaps the similar-sized polypeptide reported to copurify with LHC-II by Suss and Brecht (37).

A Western blot of antibodies from classes V and VI is shown in Fig. 4. These classes of antibodies reacted with polypeptides of both LHC-I and LHC-II. The reactions with LHC-I were verified in Western blots using purified PSI preparations (Fig. 4, lanes *e* and *h*). The antibodies of classes V and VI also tended to weakly bind various other thylakoid polypeptides that do not appear to be associated with the light-harvesting complexes. We believe that this weak binding was nonspecific since it decreased rapidly upon dilution of the antibody.

Of the 14 antibodies characterized, eight were IgG, and six were IgM. Both types of antibody were present in each class that contained more than one member (data not shown). Antibodies MLH1 and MLH2 were IgG.

Characterization of Binding Sites of MLH1 and MLH2

The class I and class II antibodies, which recognized individual polypeptides, were further characterized. To test if the epitopes that the antibodies recognized were surface exposed, Triton-solubilized LHC-II was digested with trypsin before gel electrophoresis and transfer to nitrocellulose. This treatment removes a 1–2-kD fragment from the amino terminus of the 26-kD polypeptide (30, 36). Trypsin also digests at least a 3-kD fragment from the 28-kD polypeptide (Fig. 5, *a*). Western blots with either MLH1 or MLH2 (Fig. 5, *b* and *c*) show that antibody binding is eliminated by this trypsin action. We conclude that the binding site for both antibodies is located on a surface-exposed, trypsin-cleavable

fragment. This fragment is not visible on the blot since our gel system did not resolve peptides of <8 kD. Polypeptide fragments of 17.5 and 15 kD, released by trypsin treatment, were not reactive with either antibody.

Binding assays using ^{35}S -labeled antibody indicated that the trypsin-cleavable antibody binding site on both polypeptides is exposed on the stroma side of the membrane (i.e., accessible to antibody in intact membranes). Fig. 6 shows the binding of ^{35}S -labeled MLH2 to 4 μg of unstacked, intact thylakoid membranes. Sonication of the thylakoids for 3 min with a probe tip sonicator to randomize membrane orientation did not increase the maximum level of binding (data not shown). Trypsin treatment of the intact membranes reduced the antibody bound to $<10\%$ and apparently eliminated any saturable binding (Fig. 6). Similar results were found for MLH1.

Analysis of the binding data by Scatchard plot (Fig. 7) indicated that there were 0.068 MLH1 binding sites and 0.0051 MLH2 sites per chlorophyll. This is equivalent to 27 and 2 binding sites per 400 chlorophylls for antibodies MLH1 and MLH2, respectively. Assuming that all binding sites were saturated in both cases, this represents the number of each polypeptide in the thylakoid membrane per electron transport chain (assuming 400 chlorophyll per chain; reference 20). However, since it is known that the gene family encoding LHC-II exhibits some sequence variation at the presumed antibody binding site (12), it is possible that not all 26- or 28-kD LHC-II polypeptides are recognized by the antibodies. The binding constants, K_D , were 7.4×10^{-8} M for MLH1 and 1.2×10^{-9} M for MLH2.

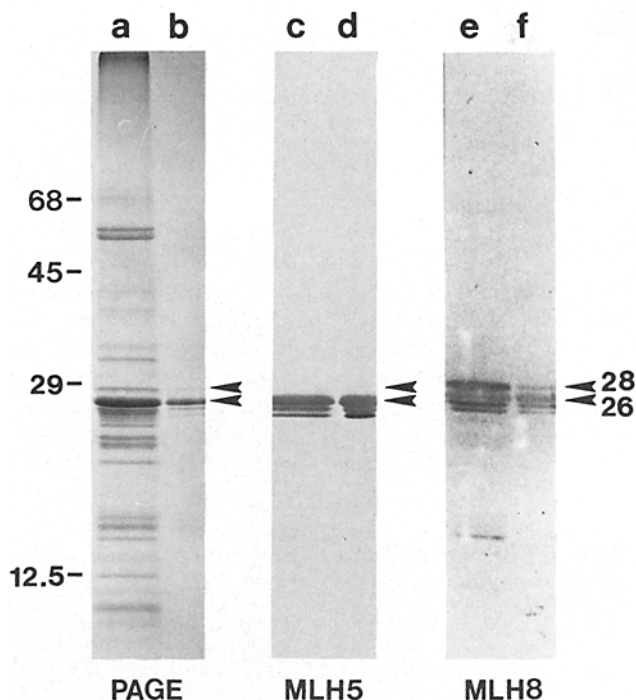


Figure 3. Western blot and stained PAGE of pea thylakoid membranes probed with class III and class IV monoclonal antibodies. Lanes *a* and *b*, Coomassie Blue-stained PAGE of pea thylakoid membranes (*a*) and isolated LHC-II (*b*). Lanes *c-f*, Western blots of thylakoid membranes (lanes *c* and *e*) and isolated LHC-II (lanes *d* and *f*) probed with the class III antibody MLH5 (lanes *c* and *d*) and the class IV antibody MLH8 (lanes *e* and *f*).

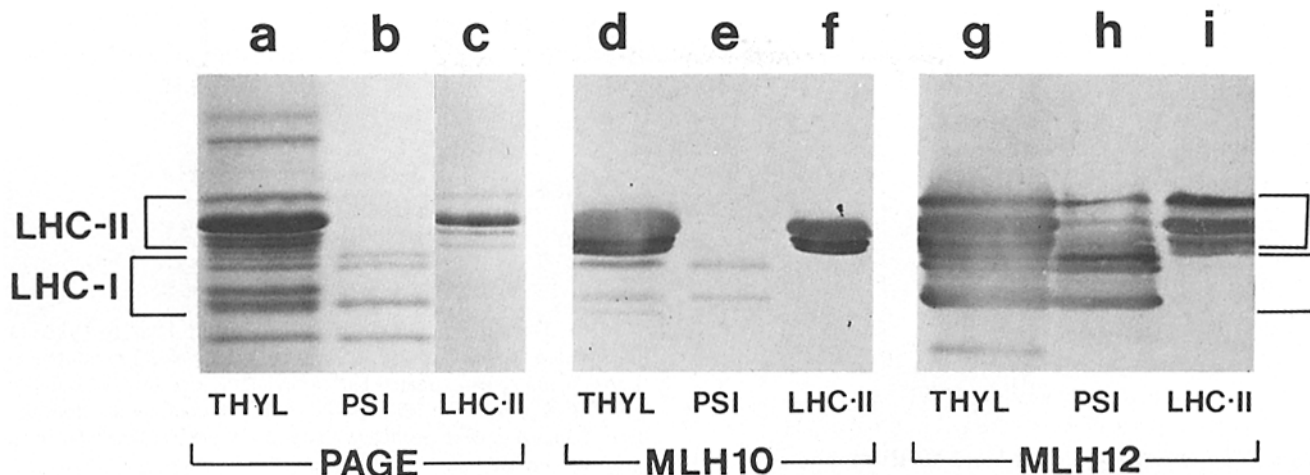


Figure 4. Western blot and stained PAGE of pea thylakoid membranes, isolated LHC-II, and isolated PSI probed with class V and VI antibodies. A close up of the LHC-II and LHC-I regions is shown. Lanes *a*, *d*, and *g*, thylakoid membranes; lanes *b*, *e*, and *h*, isolated PSI polypeptides; lanes *c*, *f*, and *i*, isolated LHC-II. Lanes *a*-*c*, stained PAGE; lanes *d*-*i*, Western blots reacted with the class V antibody, MLH10 (*d*-*f*) and the class VI antibody, MLH12 (*g*-*i*). Note that the PSI preparation used in lane *h* was contaminated with LHC-II so that both types of polypeptides appear in the lane. The molecular weights of the three LHC-I polypeptides recognized by the class V and VI antibodies were 24, 23.5, and 21.5 kD.

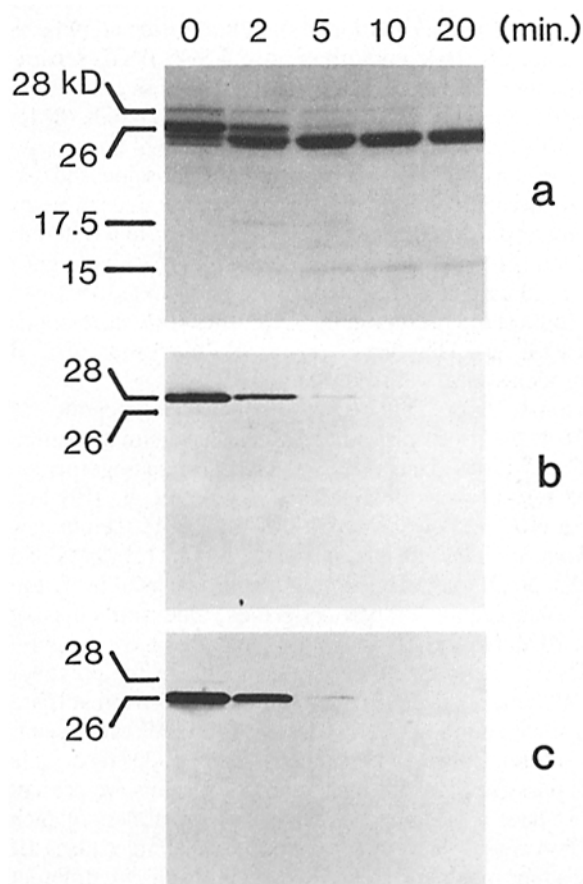


Figure 5. Time course of trypsin treatment of isolated LHC-II. (a) Stained PAGE; (b) Western blot using antibody MLH2; (c) Western blot using antibody MLH1. A time course from 1 to 20 min of trypsin digestion of isolated LHC-II is shown. In both cases the antigenic determinant is removed by trypsin digestion.

LHC-II Polypeptides in the Absence of Chlorophyll *b*

The chlorophyll *b*-less (*chlorina f2*) mutant of barley is thought to contain no functional LHC-II (6, 39). A preparation of mutant barley thylakoids was analyzed by Western blot using antibodies MLH1 and MLH2 (Fig. 8). The 26-kD polypeptide, which is greatly reduced in the membranes as indicated by Coomassie Blue staining (Fig. 8, lane *b*), was only weakly labeled by the MLH1 antibody (lane *d*). In contrast, the 28-kD polypeptide showed approximately equal labeling intensity in both mutant and wild-type thylakoids (lanes *e* and *f*). The amount of membranes that was loaded on the gel was not normalized between the wild-type and mutant membranes by any parameter other than relative intensity of non-LHC-II stained bands. Therefore, quantitative comparisons between mutant and wild-type membranes are not possible. However, it is clear that the ratio of the 26- to the 28-kD polypeptides is very much reduced in the mutant relative to that ratio in the wild-type membranes.

IML treatment of etiolated barley seedlings produces an arrested developmental state similar to that of the chlorophyll *b*-less mutant of barley. The chlorophyll *a*/chlorophyll *b* ratio of these thylakoids is high (2), and the membranes are functionally active. However the photosynthetic unit size is re-

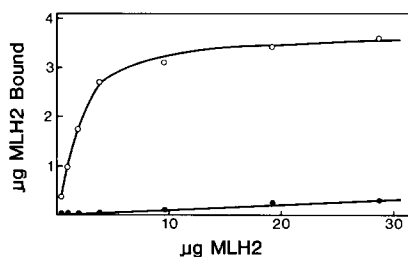


Figure 6. Binding of ^{35}S -labeled MLH2. ○, 4 μg chlorophyll of pea thylakoid membranes; ●, 4 μg chlorophyll of trypsin-treated pea thylakoid membranes. Specific activity of the antibody was 820 ± 50 dpm/ μg IgG.

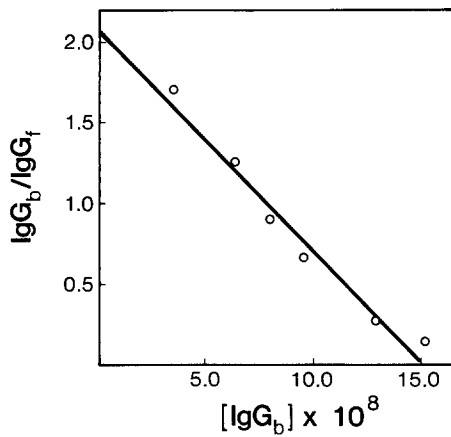


Figure 7. Scatchard plot of ^{35}S -labeled MLH1 binding to 1 μg of pea thylakoid chlorophyll. Specific activity of MLH1 was $1,380 \pm 140$ dpm/ μg IgG.

duced due to the absence of pigmented LHC-II and LHC-I complexes (3, 18, 33). Western blots of thylakoid membranes isolated from IML-treated barley seedlings yielded results that are qualitatively similar to those of the *chlorina f2* mutant. The ratio of the 26-kD to the 28-kD polypeptide was much reduced in the IML membranes relative to that in the control membranes (Fig. 9, lanes *a* and *d* vs. lanes *b* and *e*). When IML-treated seedlings are placed in continuous light, the synthesis of chlorophyll *b* is no longer suppressed and LHC-II development begins (2). In Western blots of thylakoids from IML-treated seedlings that were exposed to 4 h of continuous illumination, the amount of the 26-kD polypeptide in the thylakoids increased while the amount of the

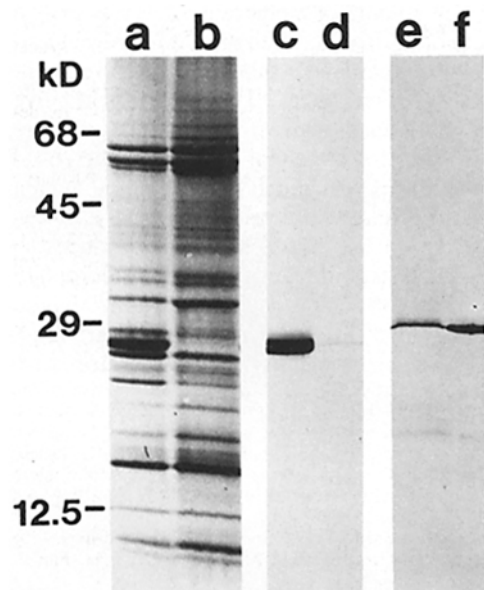


Figure 8. Stained PAGE and Western blot of mutant and wild-type barley thylakoid membranes. Lanes *a*–*b*, stained PAGE of wild-type barley thylakoid membranes (*a*); chlorophyll *b*-less mutant thylakoid membranes (*b*). Lanes *c*–*f*, Western blot of wild-type barley probed with MLH1 (*c*); chlorophyll *b*-less mutant probed with MLH1 (*d*); chlorophyll *b*-less mutant probed with MLH2 (*e*); wild-type barley probed with MLH2 (*f*).

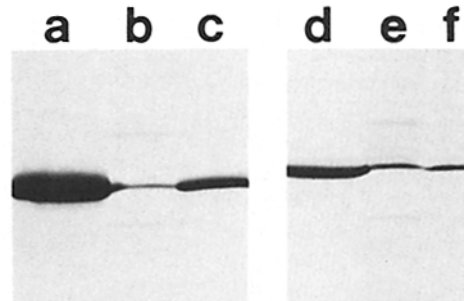


Figure 9. Western blot of IML-treated and control barley thylakoid membranes. Lanes *a* and *d*, control barley thylakoid membranes. Lanes *b* and *e*, IML-treated barley thylakoid membranes. Lanes *c* and *f*, IML-treated barley exposed to 4 h of continuous illumination. Lanes *a*–*c* were probed with antibody MLH1; lanes *d*–*f* were probed with antibody MLH2.

28-kD polypeptide remained constant (Fig. 8, lanes *c* and *f*). We conclude that the 26-kD polypeptide is affected by the IML treatment to a much greater extent than the 28-kD polypeptide.

Discussion

Polypeptide Diversity in LHC-II

Advancements in the isolation of light-harvesting complexes of PSI and PSII, coupled with improved SDS PAGE separation of proteins, have permitted the identification of multiple polypeptides in the LHC-II of chloroplast thylakoids (8, 15, 27, 28, 30, 35, 37). The functional importance of these polypeptide species in LHC-II remains unknown, as does the origin of apparent differences in size. The various sized proteins could correspond to different gene products or to a differential pattern of posttranslational processing of a single gene product. Chua and colleagues have demonstrated that two gene products are present in the LHC-II of *Chlamydomonas* and pea (9, 35). However it is possible that other LHC-II gene products have yet to be identified.

Previously Apel (1) has shown that two polypeptides of LHC-II in the green alga *Acetabularia* have similar amino acid compositions and two-dimensional, tryptic fingerprints. Similar results were obtained by Hooper et al. (19) and Schmidt et al. (35) working with the thylakoid membranes of *Chlamydomonas* and pea, respectively. Our results extend these data to all four LHC-II polypeptides isolated by Triton X-100 solubilization of pea thylakoids. The antibodies of classes III and IV clearly show that some regions of the polypeptides are identical among three or four of the polypeptides. We believe that at least four antigenic determinants are shared since antibody classes III, IV, V, and VI each recognize a different subset of the antenna polypeptides (see Table I). It is possible that additional common regions are present since we have not determined whether all members of each class bind exactly the same epitope (for example, class III contains four members which may each recognize a different epitope).

Monoclonal antibodies of class I and class II clearly discriminate between the 28- and 26-kD polypeptides of LHC-II (Table I, Figs. 1 and 2). This eliminates the possibility that the two proteins are simply different sized derivatives of a

single cytoplasmically synthesized precursor protein. If this were the case, then all regions of the 26-kD polypeptide should repeat the primary sequence of the 28-kD polypeptide. However, MLH1 binds an epitope of the 26-kD polypeptide that is not present in the 28-kD polypeptide. This leads us to conclude that the 26-kD polypeptide is not a product of proteolytic processing of the 28-kD polypeptide. We recognize that it is possible that MLH1 binds a portion of the 26-kD polypeptide that has been posttranslationally modified after it was processed from the 28-kD polypeptide. However, we view this possibility as unlikely, especially in light of the existence of two additional antibody groups, classes III and V, which each bind other regions of the 26-kD polypeptide not present in the 28-kD polypeptide.

Our studies did not identify any antibodies that could distinguish the 25.5- and 24.5-kD proteins from the 26-kD polypeptide of LHC-II. A likely interpretation of these data is that the three proteins are derived from a common precursor protein. The epitope recognized by antibody MLH1, which is present only in the 26-kD protein, is located on the trypsin-cleavable N terminus (30; and Fig. 5) of the protein. The fact that this epitope is not present in the two smaller LHC-II polypeptides suggests that an N-terminal processing step is giving rise to the smaller proteins. Recently Kohorn et al. have found evidence for processing of the major LHC-II polypeptide (22). An LHC-II polypeptide, produced *in vitro* from a cDNA clone, was taken up by isolated intact *Lemna* chloroplasts and processed to produce several polypeptides of lower molecular weight which were incorporated into thylakoid membranes.

Common Antigenic Determinants among LHC-II and LHC-I Polypeptides

The ability of monoclonal antibodies in classes V and VI to react with several polypeptides in both LHC-II and LHC-I reveals a previously unrecognized similarity in these polypeptides. We suspect that the lack of evidence for this similarity in previous immunological studies is caused by the nature of the common antigenic determinant. This peptide region could either be weakly antigenic and/or antibodies binding it could have low affinity. As a result, this class of antibodies would be difficult to detect in a polyclonal preparation. However, several laboratories have recently observed cross-reactivity between LHC-II and LHC-I in their polyclonal antibodies (White, M., and B. Green, University of British Columbia, personal communication; and Schmidt, G., University of Georgia, personal communication). The fact that there is commonality among polypeptides of LHC-II and LHC-I may not be surprising since both complexes have similar functions in binding the antennae pigments, chlorophyll *a*, chlorophyll *b*, and carotenoids. These limited data do not allow any further speculation, however, about the extent of the similarities or origins of the genes encoding these polypeptides.

Developmental Variation in Stoichiometry of LHC-II Polypeptides

The use of antibodies MLH1 and MLH2 allowed relative quantitation of the 28- and 26-kD polypeptides in mutant and partially developed thylakoid membranes that were devoid or depleted of chlorophyll *b* (Figs. 7 and 8). In both cases the amount of the 26-kD protein was greatly reduced, whereas

the 28-kD polypeptide was at near normal levels. This result extends the work of others (4, 10, 34) who have found small pools of the 26-kD apoprotein present in these two types of thylakoid membranes. We conclude that assembly of the 28-kD polypeptide is not regulated by chlorophyll *b* binding as has been shown for the 26-kD polypeptide (4, 10). This suggests that the 28-kD polypeptide may not bind chlorophyll *b* in the thylakoid membrane.

In summary, we have reported the preliminary characterization of a collection of 14 monoclonal antibodies to LHC-II. These antibodies add support to the hypotheses that at least two distinct gene products are present in LHC-II (35) and that some LHC-II polypeptides may arise from posttranslational processing of a single precursor. The characterization also revealed a group of antibodies that cross-react with polypeptides of both LHC-II and LHC-I, indicating that there are structural similarities between the two types of antenna complex. Finally, two antibodies from the collection have allowed us to distinguish between and quantify the 26- and 28-kD polypeptides. The two polypeptides are unique components of LHC-II which respond to the absence of chlorophyll *b* differently. We believe that these two polypeptides may comprise separate structural domains in the LHC-II antenna complex.

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