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The PAS2 domain is required for dimerization of phytochrome A

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Abstract

Phytochromes are plant photoreceptors that regulate the growth and development of plants in response to light. Phytochromes exist as dimers and dimerization is thought to be important for phytochrome function. Phytochromes contain two major domains, the N-terminal domain responsible for chromophore ligation and photosensory specificity and the C-terminal domain responsible for dimerization and regulatory functions. We have investigated the dimerization motifs by means of the yeast two-hybrid assays and size exclusion chromatography using purified recombinant phytochromes. From dimerization analyses using internal deletion mutants, site-specific mutants, and C-terminal fragments of the pea phytochrome A, the primary contact region for dimerization was localized to the region between Val730 and Gly821. Further analysis using purified full-length phytochrome mutants and Per–Arnt–Sim 1 (PAS1) and PAS2 fragments revealed that the PAS2 domain is required for dimerization, but that the PAS1 domain is not.

Keywords: Phytochrome; Dimerization; PAS domain; PAS2

1. Introduction

Plant phytochromes are red and far-red photoreceptors that regulate various aspects of plant growth and development in response to light signals [1–3]. Phytochromes are dimers consisting of two ~120 kDa subunits [4]. Each subunit consists of two major domains: a globular N-terminal domain (65 kDa), which is required for chromophore attachment and spectral integrity, and a more linear C-terminal domain (60 kDa), which mediates dimerization and interactions with signaling partners [5,6]. Although the molecular mechanism and biological function of dimerization is not well-understood, studies indicate that dimerization is crucial for phytochrome action [7–9]. Recently, the N-terminal domain of phytochrome B (phyB) was shown to be functional in vivo, when dimerized and localized in the nucleus by means of motifs added to the N-terminal domain construct [10,11]. These results suggest that in the native protein the C-terminal domain mediates dimerization and regulatory function, such as nuclear localization. Moreover, heterodimerizations of photostable type II phytochromes have been detected in *Arabidopsis* [12], emphasizing the importance of dimerization in modulating phytochrome-mediated signal transduction in plants.

The C-terminal domain of pea phytochrome A (phyA) contains a Per–Arnt–Sim (PAS)-related domain consisting of a pair of PAS repeats (PAS1 and PAS2) and a histidine kinase-related domain (HKRD) (see Fig. 1a) [13,14]. Since PAS domains have diverse functions, including dimerization, protein–protein interactions, transcriptional activation, and cofactor binding [15–17], the PAS motifs in the C-terminal domain may be responsible for phytochrome dimerization [6]. Two PAS domains, PAS1 (G611–T736) and PAS2 (D734–S876), have been identified in oat phyA as an example. Several C-terminal regions have previously been proposed to mediate dimerization in phytochromes. Trypsin digestion of purified pea phyA releases dimeric fragments that extend from Ala753 (A753) to Arg1089 (R1089), which suggests that this region (753–1089aa) contains the sequence required for dimerization [18]. Characterization of transgenic tobacco plants expressing phytochromes with various truncations in the C-terminal domain implicated

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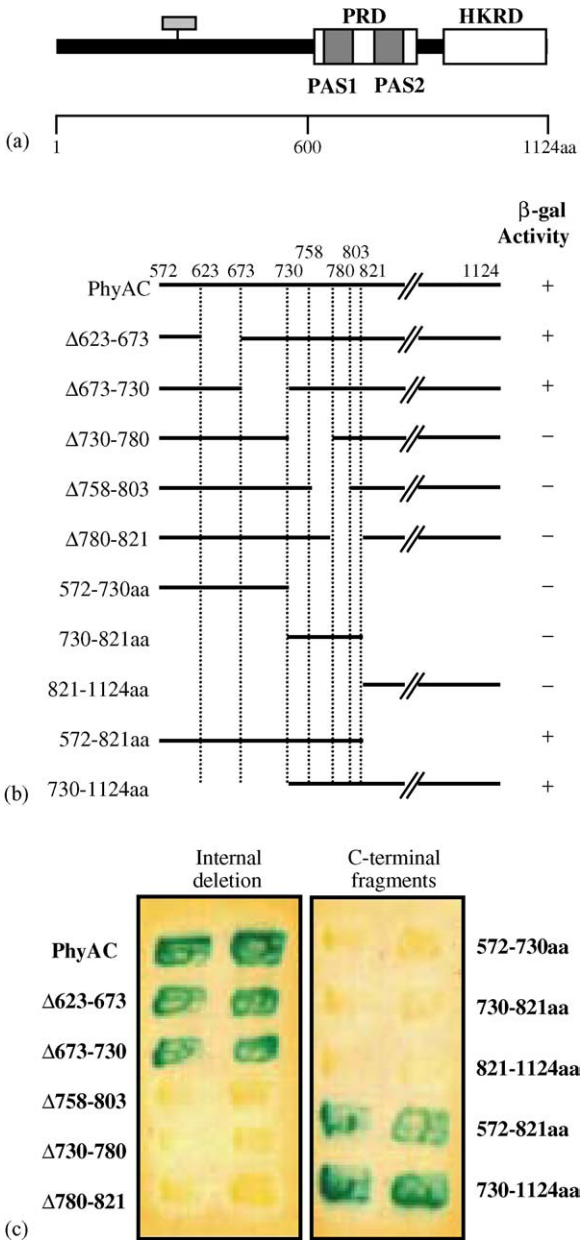

Fig. 1. Dimerization analysis of internal deletion mutants and C-terminal fragments by yeast two-hybrid assays. (a) Domain structure of the pea phyA monomer. PRD, PAS-related domain; HKRD, histidine kinase-related domain; PAS1, the first PAS repeat; PAS2, the second PAS repeat. The chromophore (rectangle) is attached at amino acid 323 in the N-terminal domain. (b) Internal deletion mutants and C-terminal fragments used in these assays. Solid lines show the cloned C-terminal protein sequence and deleted regions are shown as a blank. (c) Dimerization assays with internal deletion mutants and C-terminal fragments of pea phyA. The C-terminal domain (572–1124aa) of wild-type pea phyA, five internal deletion mutants (Δ623–673 through Δ780–821), and five different C-terminal fragments (572–730aa through 730–1124aa) were subcloned into both pAS2-1 and pACT2 vectors. The β-galactosidase activity (blue colony color) of co-transformed yeast cells was determined as a measure of dimerization ability of each mutant.

amino acids 919–1093 in phytochrome dimerization [9]. Edgerton and Jones [19] carried out a dimerization study of oat phyA using an *Escherichia coli* in vivo two-hybrid assay based on protein fusions with a lambda repressor and proposed amino acids 623–673 as the region of primary contact. Subsequently, these researchers proposed that amino acids 599–683 form the critical region for phytochrome dimerization based on dimerization assays using in vitro translated phytochrome fragments [20]. However, the results indicate that neither the 623–673aa nor the 599–683aa domains are sufficient to mediate dimerization. Moreover, since deletion of a similar region in phyB (amino acids 652–712) does not affect dimerization when expressed in transgenic *Arabidopsis* [9], this region does not appear to be necessary for the dimerization of native phytochromes.

Romanowski and Song [21] performed a structural domain identification of phyA molecules by auto-correlation analysis of the amino acid sequences of homologous phytochromes. They proposed an antiparallel β-strand model for the phyA dimer. Analysis of six phyA species, including those from oat and pea, indicated that the dimerization region is located in residues 730–821. These previous studies have implicated several different regions within amino acids 623–821, including two PAS domains (PAS1 and PAS2), as the dimerization domain of phyA. However, the exact dimerization domain is still controversial because the crystal structure of the phytochrome dimer has not been solved.

In the present study, we surveyed dimerization domains in vivo by yeast two-hybrid assay using internal deletion mutants and C-terminal domain fragments of phyA, and in vitro by size exclusion chromatographic analysis of purified recombinant mutant phytochrome proteins. Our results indicate that the PAS2 domain is primarily responsible for the dimerization of phyA.

2. Materials and methods

2.1. Phytochrome gene constructs

Five different internal deletion mutants and five different fragments of the C-terminal domain of pea phyA (see Fig. 1b) were subcloned into the Matchmaker two-hybrid system 2 (Clontech, Palo Alto, CA) for yeast two-hybrid assays (see Fig. 1a). All mutant genes were generated by polymerase chain reaction (PCR) and subcloned into the *Nco*I and *Bam*HI sites (in the pAS2-1 and pACT2 vectors). For the Δ623–673 construct (C-terminal domain (572–1124aa) with a deletion from R623 to T673), two primer sets were used. The primers, 5′-CTCCCATGGATGCTATTCATTCGTTGC-3′ (*Nco*I, forward; Primer #1) and 5′-GCCCCGGGAACCATCTCACTTGTCACTGC-3′ (*Xma*I, reverse) were used for the 572–622aa fragment, and 5′-CTCCCCGGGGATATAGTCAAGAAGATGAG-3′ (*Xma*I, forward) and 5′-GCGGATCCTCATTTCAACTTATGAGCTGCTG-3′ (*Bam*HI, reverse; Primer #2) were used for the 674–1124aa fragment. The underlined nucleotide sequences show the restriction sites used for subcloning. The two fragments were ligated into the pAS2-1 and pACT2 vectors digested with *Nco*I and *Bam*HI. Clones were screened by restriction digest and confirmed by DNA sequencing.

All other internal deletion constructs were generated by the same method using the following primers: for the Δ673–730

construct, Primer #1 and 5′-GCCCCGGGTGAAGAATCCTC-AACCAGGG-3′ (*Xma*I, reverse), and 5′-CTCCCCGGGGC-CCAAGATATAACTGCTCAG-3′ (*Xma*I, forward) and Primer #2; for the Δ730–780 construct, 5′-GCCCCGGGAAAACA-AACCCCCACTAC-3′ (*Xma*I, reverse) and 5′-CTCCCCGGG ATGATTAAGTTAACCGGATGG-3′ (*Xma*I, forward); for the Δ758–803 construct, 5′-GCCCCGGGGTTCTGCACAATTG-CTTTGTAATCG-3′ (*Xma*I, reverse) and 5′-CTCCCCGGG ACTCAAATGTCTTGTTGTCGTC-3′ (*Xma*I, forward); for the Δ780–821 construct, 5′-GCCCCGGGTGCATTCCACTCA-CAACACC-3′ (*Xma*I, reverse) and 5′-CTCCCCGGGATTG-TACTTAATAAAGCCATGAC-3′ (*Xma*I, forward). The C-terminal fragments were also generated by PCR with the following primers: Primer #1 and 5′-GCGGATCCCACAAAA-CAAACCCCCAC-3′ (*Bam*HI, reverse) for 572–730aa, 5′-CTC CCATGGGTGGCCCAAGATATAACTGC-3′ (*Nco*I, forward; Primer #3) and 5′-GCGGATCCGCCGAAATTAACAAAA-GCTTC-3′ (*Bam*HI, reverse; Primer #4) for 730–821aa; 5′-CTCCCATGGGGCATTGTACTTAATAAAGCCATG-3′ (*Nco*I, forward) and Primer #2 for 821–1124aa; Primer #1 and #4 for 572–821aa; Primer #3 and #1 for 730–1124aa.

In order to create G1 and G2 mutants, site-directed mutagenesis was performed using the GeneEditor™ in vitro site-directed mutagenesis system (Promega, Madison, WI), according to the manufacturer's protocol. The G1 mutant contained four substitutions: K738V, D742V, R747V, and D750V. The G2 mutant contained five substitutions: K788V, R789L, E790V, E791V, and K795V.

2.2. Yeast transformation

The yeast strain used for yeast two-hybrid assays was *Saccharomyces cerevisiae* Y187 (*MATa*, *ura 3–52*, *his 3–200*, *ade 2–101*, *trp 1–901*, *leu 2–3*, *112*, *gal4D*, *met*, *gal 80D*, *URA3::GAL1UAS-GAL1TATA-lacZ*) (Clontech, Palo Alto, CA). Yeast transformation was carried out using alkali–cation yeast transformation kits (Bio 101, La Jolla, CA), according to the manufacturer's instructions. The transformed cells were screened on leucine and tryptophan deficient minimal medium plates. Because pAS2-1 has the *trp* gene and pACT2 has the *leu* gene as selection markers, only co-transformed Y187 cells with mutant constructs in pAS2-1 and pACT2 can survive in the leucine and tryptophane deficient minimal medium. Each co-transformed yeast clone was double screened on the same minimal medium and the expression of phytochrome proteins was examined by Western blot analysis (see below) before dimerization assays were carried out.

2.3. Dimerization assay by yeast two-hybrid

A colony-lift filter assay was used to test dimerization of phyA mutants. Fresh, well-established colonies were used for the assays. A clean, dry filter (Whatman #5) was placed over the surface of the agar plate containing colonies to be assayed. The carefully lifted filter was submerged in liquid nitrogen for 20 s. After the filter was completely frozen, it was removed from the liquid nitrogen and thawed at room temperature. This freeze–thaw treatment was repeated to permeabilize the cells. The completely thawed filter was placed colony-side up on two sheets of filter paper presoaked with Z-buffer/X-gal solution. After an approximately 10 h incubation at 30 °C, blue colonies expressing mutant phytochromes that were able to dimerize were observed. The Z-buffer contained $Na_2HPO_4{\cdot}7H_2O$ (16.1 g/L), $NaH_2PO_4{\cdot}H_2O$ (5.5 g/L), KCl (0.75 g/L) and $MgSO_4{\cdot}7H_2O$ (0.246 g/L). The concentration of X-gal stock solution was 20 mg/mL in *N*,*N*-dimethyl formamide. The Z-buffer/X-gal solution consisted of 100 mL Z-buffer, 0.27 mL β-mercaptoethanol and 1.67 mL X-gal stock solution.

2.4. Preparation of full-length recombinant pea phyA proteins

To study dimerization by size exclusion chromatography, two internal deletion mutants and one site-specific mutant, as well as the wild-type pea phyA gene were subcloned into the pPIC3.5K vector (Invitrogen, Groningen, The Netherlands) for the *Pichia* expression system, as previously described [22]. The primers, 5′-CGGGATCCACCATGGCAACCACGAGGCCTAGCC-3′ (forward, *Bam*HI) and 5′-TCGCGTCGACCTTTCAACTTA-TGAGCTGC-3′ (reverse, *Sal*I) were used for cloning of full-length pea phy A genes into pPIC3.5K. The pPIC3.5K constructs were transformed into *Pichia* using a Micropulser™ Electroporation apparatus (Bio-Rad, Hercules, CA) and recombinant phytochrome proteins expressed, according to the manufacturer's recommendations (Invitrogen, Groningen, The Netherlands). The expression levels in *Pichia* were very low (less than 0.1 mg/mL of culture) and the purified proteins, therefore, concentrated using Centricon® filters to size exclusion chromatography.

2.5. Dimerization assay by size exclusion chromatography

Size exclusion chromatography of pea phyA mutant proteins was performed using a Shimadzu LC-10AT HPLC system with a Bio-Sil SEC 250–5 column (Bio-Rad, Hercules, CA) with the mobile phase (50 mM Tris–HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl) pre-filtered through a 0.4 μm HA filter (Millipore) and a flow rate of 0.5 mL/min. Analysis of oat phyA PAS domains used an ÄKTA FPLC system with a variable wavelength UV–visible detector with a Sephadex® 75 HR 10/30 column (Amersham Biosciences, Seoul, Korea) equilibrated with the size exclusion chromatography buffer (10 mM Tris–HCl, pH 7.8, 150 mM NaCl, 2 mM DTT) at 4 °C and a flow rate of 0.5 mL/min. Molecular size calibration was carried out using Bio-Rad gel filtration standards (Cat. No. 151–1901) containing bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and Vitamin B12 (1.35 kDa) under the same conditions.

The eluted fractions (0.5 mL) were analyzed by SDS-PAGE and Coomassie staining or Western blotting. For Western blot analysis, proteins on SDS-PAGE gels were transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond–P^+;

Amersham Biosciences, Seoul, Korea) overnight at 30 V (or for 1 h at 100 V). Pea phyA-specific monoclonal antibodies (mAP10 and mAP20) [23] were used to identify recombinant phytochrome proteins, and mouse peroxidase-conjugated secondary antibody was used for detection by ECL™ (Amersham Biosciences, Seoul, Korea), according to the manufacturer's protocol.

2.6. Preparation of PAS domains of recombinant oat phyA proteins

The PAS1 (G611–T736) and PAS2 (D734–S876) domain DNAs of oat phyA were amplified by PCR with the following primers: 5′-CACGGATCCGGGCTTGCTGAACTGC-3′ (*Bam*HI, forward) and 5′-ATAGAATTCTCAAGTCATATCTTGGGCAAC-3′ (*Eco*RI, reverse) for PAS1, and 5′-CACGGATCCGATATGACTGTCCATAAG-3′ (*Bam*HI, forward) and 5′-ATAGAATTCTCAACTAGCAACATGAATAAAAC-3′ (*Eco*RI, reverse) for PAS2. After amplification, the DNA fragments were purified and digested with *Bam*HI and *Eco*RI, ant then inserted into the pGEX-4T-2 plasmid digested with the same enzymes. PAS1 and PAS2 DNA constructs were transformed into *E. coli* BL21 (DE3) cells. Glutathione-*S*-transferase (GST) fused to PAS proteins was used for purification. For protein preparations, an 80 mL overnight culture (37 °C) in 2× YTA medium (per liter: 16 g tryptone, 10 g yeast extract, and 5 g NaCl, pH 7.0) containing 100 μg/mL ampicillin was used to inoculate 4 L of the same medium. The culture was incubated at 22 °C for 7 h. The temperature was lowered to 18 °C and IPTG (final concentration 0.1 mM) was added to induce expression of the proteins. Cultures were incubated overnight and cells were then harvested and resuspended in 200 mL of ice-cold PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Cells were disrupted by sonication and then incubated with Triton X-100 (final concentration 1%) for 30 min at 4 °C. After centrifugation (26,000 × *g*, 45 min, 4 °C) the lysate was incubated with 2 mL of glutathione sepharose 4B resin for 1 h at room temperature. After incubation, the resin was loaded into a column and thoroughly washed with PBS buffer at 4 °C. GST–PAS was eluted by application of four 5 mL aliquots of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0). The protein was concentrated to 3 mL and incubated with 120 units of thrombin for 16 h at 25 °C. After thrombin digestion, the protein was dialysed against PBS buffer and the GST tag removed with equilibrated glutathione gel. The PAS protein was diluted with 50 mM Na–HEPES (pH 7.2) buffer and applied to a DEAE column equilibrated with the same buffer. After washing, a gradient (0–0.5 M NaCl) was applied, the PAS-containing fractions were pooled and concentrated to 0.5 mL. The final purification step and oligomeric state determination for PAS constructs used size exclusion chromatography.

3. Results and discussion

Since previous work has suggested that the dimerization region of phytochromes resides in the C-terminal domain and potentially in the PAS domains, we first decided to define the dimerization regions in the C-terminal domain using internal deletion mutants of pea phyA (Fig. 1a). Approximately 50 amino acids were deleted from different segments of the phytochrome C-terminal domain (Fig. 1b). Mutants Δ623–673, Δ673–730, Δ730–780, Δ758–803, and Δ780–821 were chosen for analysis because these regions overlap most of the proposed dimerization sites. We included the full-length wild-type C-terminal domain (572–1124aa, phyAC) of pea phyA as a positive control. The ability to dimerize was determined by yeast two-hybrid assays. Since this two-hybrid interaction system is based on in vivo protein–protein interactions, the proteins are most likely to be in their native conformations. Dimerization was detected by *lacZ* gene expression because activation of the reporter gene (β-galactosidase) occurs only when the cloned mutant phytochromes dimerize. These assays showed that dimerization of Δ623–673 and Δ673–730 was comparable to the wild-type (phyAC), but dimerization was abolished in the Δ758–803, Δ730–780, and Δ780–821 mutants (Fig. 1c, left), indicating that amino acids 730–821 are required for dimerization.

We further analyzed dimerization using five different fragments of the C-terminal domain, including 572–730aa, 730–821aa, 821–1124aa, 572–821aa and 730–1124aa. Based on the internal deletion results above, we predicted a positive dimerization signal from the 730–821aa fragment and negative signals from the 572–730aa and 821–1124aa fragments. However, none of the fragments were able to dimerize (Fig. 1c, right), indicating that the 730–821aa fragment is not sufficient for dimerization. We then tested the 572–821aa and 730–1124aa fragments to probe the involvement of the 730–821aa region. Both fragments were able to dimerize (Fig. 1c, right). From these data, we suggest that amino acids 730–821 are primarily involved in phytochrome dimerization, but are not sufficient for dimerization.

The C-terminal domain of phyA contains a pair of PAS repeats (PAS1 and PAS2) and a histidine kinase domain. In pea phyA, PAS1 spans G610–T735 (610–735aa) and PAS2 spans D733–S875 (733–875aa). Given that the PAS domains have been implicated in dimerization [15], our results suggest that PAS2 serves as the primary dimerization domain of pea phyA. Recently, PAS1 was shown to mediate protein–protein interaction between phyA and nucleotide diphosphate kinase 2 (NDPK2) [24]. Another mutation in the PAS1 domain, V631M of *Arabidopsis* phyA, was reported to form dimers but disrupted phyA signaling [25], which is consistent with our results that PAS1 is not involved in dimerization. Thus, it is likely that PAS1 is involved in protein–protein interactions other than dimerization, and that PAS2 mediates dimerization.

To determine whether any specific subdomains or residues among amino acids 730–821 were involved in dimerization, two site-specific mutants were generated based on secondary structure analysis of the proposed dimerization region by the Protein Sequence Analysis and Modeling (pSAAM) program (Crofts, IL). Residues 700–860 strongly favor a surface β-strand structure and two surface β-strands were predicted: amino acids 735–750 (g1) and amino acids 780–800 (g2) (Fig. 2a). These predicted surface β-strands overlap the regions (735–765 and 790–816) that have been proposed to be two antiparallel β-

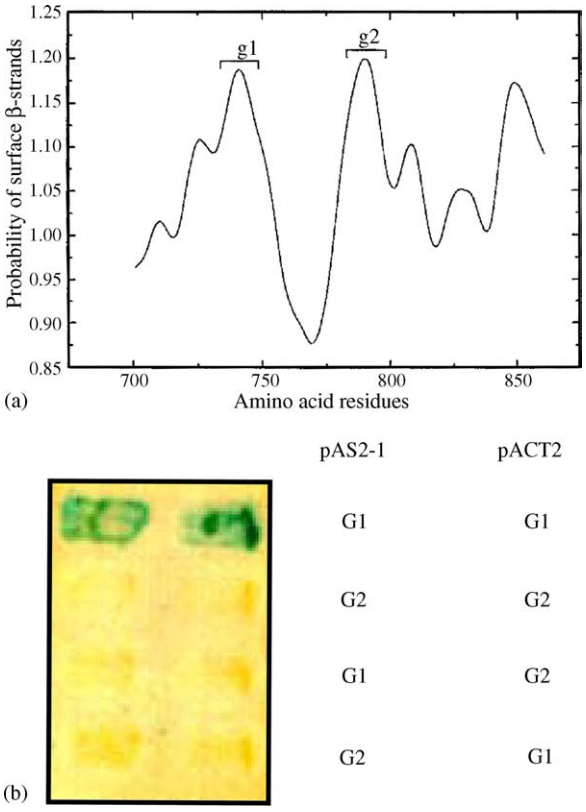

Fig. 2. Dimerization assays with site-specific mutants. (a) Predicted secondary structure of the C-terminal region containing the proposed dimerization segments (amino acids 700–860) by the pSAAM prediction program. Two regions were strongly predicted as surface β-strands, amino acids 735–750 (g1) and 780–800 (g2). (b) Dimerization assays of site-specific G1 and G2 mutants by yeast two-hybrid assays. G1 (K738V, D742V, R746V, and D750V) and G2 (K788V, R789L, E790V, E791V, and K795V) were subcloned into both pAS2-1 and pACT2, and dimerization assays were performed, as in Fig. 1.

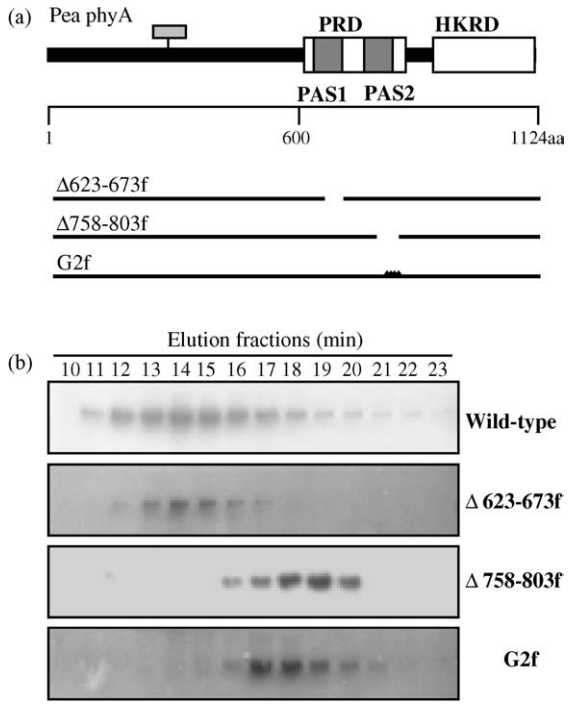

Fig. 3. Dimerization analysis of full-length phyA mutants by size exclusion chromatography. (a) Pea phyA mutant constructs are as follows: Δ623–673f, full-length pea phyA with deletion of amino acids 623–673; Δ758–803f, full-length pea phyA with deletion of amino acids 758–803; G2f, full-length pea phyA with G2 mutation (K788V, R789L, E790V, E791V, and K795V). (b) Size exclusion analysis of full-length phyA mutants. Chromatography was performed using a Shimadzu LC-10AT HPLC with a Bio-Sil SEC 250-5 column at a flow rate of 0.5 mL/min. Wild-type phyA provided a positive control of phytochrome dimers, and the eluted fractions were analyzed by Western blot analysis with phyA-specific antibodies. The PAS1-disrupted Δ623–673f mutant still dimerized, while the PAS2-disrupted Δ758–803f and G2f mutants eluted as monomers.

strands for phytochrome dimerization [21], and are strongly conserved among phyA and phyB. Since ionic interactions may be involved in phytochrome dimerization [21], we generated site-specific mutants of the g1 and g2 motifs in which the charged amino acids were changed to neutral residues. The G1 mutant was composed of the full-length C-terminal domain with substitutions of Lys-738, Asp-742, Arg-747, and Asp-750 to Val (K738V, D742V, R747V, and D750V). The G2 mutant was composed of the full-length C-terminal domain with substitutions of Lys-788, Glu-790, Glu-791, and Lys-795 to Val and Arg-789 to Leu (K788V, R789L, E790V, E791V, and K795V). The G1 mutant was able to dimerize, but the G2 mutant was not (Fig. 2b). When the G1 and G2 mutants were combined, they gave negative signals in either orientation. Amino acid sequence comparison indicated that the residues in the g2 motif were highly conserved compared to other C-terminal regions. The data are consistent with the results of the internal deletion and truncation mutants. Thus, we suggest that the β-strand of g2 in the PAS2 domain mediates phytochrome dimerization.

To confirm that PAS2 is the primary dimerization domain of phyA, we prepared recombinant proteins of full-length phyA mutants, including Δ623–673f as a PAS1 mutant, and Δ758–803f and G2f as PAS2 mutants (Fig. 3a). These pea phyA mutants were expressed using the *Pichia* expression system. Recombinant phyA mutants were purified, concentrated, and fractionated using a Bio-Sil SEC 250-5 column. The eluted fractions were analyzed by Western blot. The Δ623–673f mutant dimerized, but the Δ758–803f and G2f mutants existed as monomers (Fig. 3b). These results are consistent with the yeast two-hybrid assays. Because the PAS2 domain was impaired in the Δ758–803f and G2f mutants, these results confirmed that PAS2 was responsible for phytochrome dimerization.

We also investigated the quaternary structure of the PAS1 and PAS2 domains directly. For this analysis, we subcloned the PAS1 (G611–T736) and PAS2 (D734–S876) domains of oat phyA into the pGEX vector and their GST-fused recombinant proteins were prepared in *E. coli*. After removal of the GST tag, the PAS1 and PAS2 proteins were analyzed by size exclusion chromatography using a Sephadex® 75 HR 10/30 column. The theoretical molecular masses of PAS1 and PAS2 are 13.6877 kDa and 15.8951 kDa, respectively. Thrombin digestion of the GST tag leaves two extra amino acids at the N-terminus, Gly, and Ser. Thus, the calculated molecular masses of PAS1 and PAS2 are 13.8318 kDa and 16.0392 kDa. The size exclusion column was calibrated with with standards including chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and

Vitamin B12 (1.35 kDa), which eluted at 10.151 mL, 12.424 mL, and 18.493 mL, respectively. The void volume was 7.5 mL with blue dextran. To estimate molecular masses, we plotted the log molecular masses versus V_e/V_o, where V_e is the elution volume and V_o is the void volume. We obtained a calibration standard line of $Y = -1.36X + 6.48$, where Y is the log molecular mass and X is V_e/V_o, and $r^2 = 0.99999$. The recombinant PAS1 fragment eluted at 12.527 mL and the estimated molecular mass was 16.3 kDa (Fig. 4a and b), while the PAS2 fragment eluted at 7.878 mL and its estimated molecular mass was >44 kDa (Fig. 4c and d). The estimated molecular mass of PAS1 (16.3 kDa) is slightly higher than the calculated molecular mass (13.8 kDa), which may due to its somewhat extended structure. The elution profile suggests that PAS2 exists as dimers or higher order aggregates.

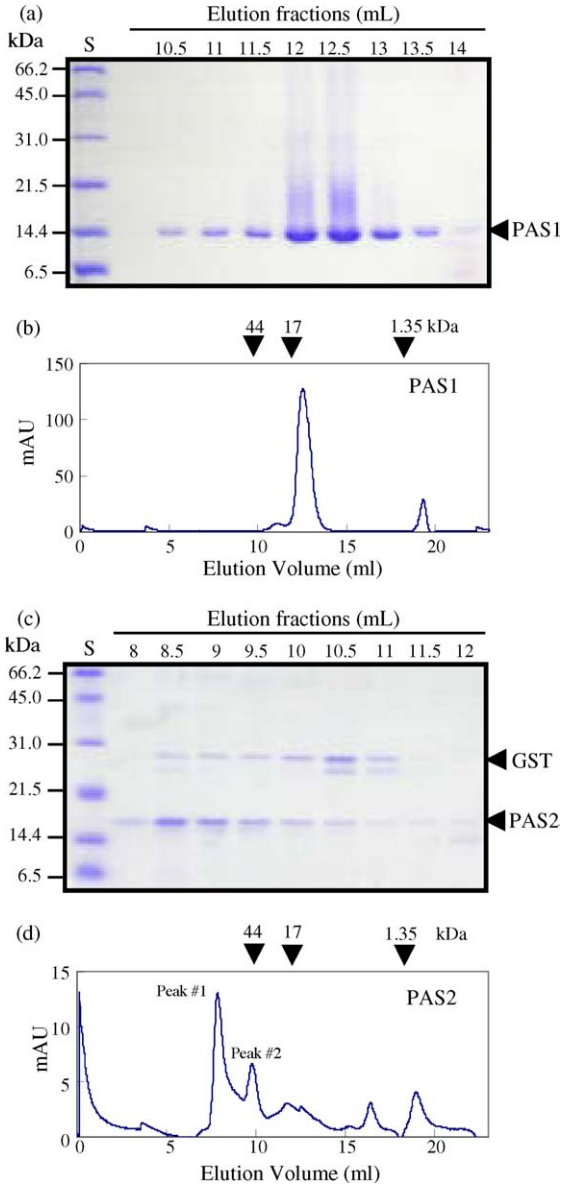

Fig. 4. Dimerization analysis of PAS1 and PAS2 by size exclusion chromatography. (a) SDS-PAGE of eluted fractions of purified PAS1. (b) Size exclusion chromatogram of PAS1. PAS1 eluted at 12.527 mL. (c) SDS-PAGE of eluted fractions of purified PAS2. (d) Size exclusion chromatogram of PAS2. PAS2 eluted at 7.878 mL (Peak #1), while GST eluted at 9.815 mL (Peak #2). The standards (arrowheads) were eluted as follows: 44 kDa at 10.151 mL, 17 kDa at 12.424 mL, and 1.35 kDa at 18.493 mL, and the void volume was 7.5 mL with blue dextran. The estimated molecular masses of PAS1 and PAS2 were 16.3 kDa and >44 kDa, respectively, which suggests that PAS1 exists as a monomer and PAS2 exists as aggregates. The estimated molecular mass of GST (Peak #2) was 50.6 kDa, close to that of GST dimers.

Thus, our current data suggest that some phytochrome missense mutants in the PAS2 domain could be explained by phytochrome dimerization. For example, the Glu812Lys (E812K) mutant of phyB which lies in the PAS2 domain showed fast dark reversion, resulting in deficiency of phyB activity in plants [26]. Another example is the phyA Glu777Lys (*phyA-302*) mutant in PAS2 displays an impaired subcellular localization [27]. These examples are consistent with a model in which dimerization through the PAS2 motif is required for phytochrome function.

4. Conclusions

Phytochrome A exists as a homodimer, and dimerization is thought to be required for function. Here, the primary dimerization domain for phyA was delineated by yeast two-hybrid analysis using phyA internal deletions, truncations, and site-specific mutants, and by size exclusion chromatographic analysis of full-length mutant phyA and PAS fragments. Our results suggest that PAS2 is crucial for dimerization of phyA, while PAS1 is not involved. The most likely dimerization motifs involve the β-strand within the PAS2 domain that specifically includes the sequence V730–G821. These results further our understanding of the role of dimerization in phytochrome function.

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