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Characterization of Phytochrome-Regulated Gene Expression in a Photoautotrophic Cell Suspension: Possible Role for Calmodulin

ERIC LAM,† MARK BENEDYK, AND NAM-HAI CHUA*

Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, New York 10021

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A photoautotrophic suspension culture of soybeans was found to exhibit light-dependent expression of the genes encoding the major chlorophyll *a*- and *b*-binding protein (*CAB*). The expression was mediated by phytochrome, since it was induced by red light and reversed by far-red light. The maximal level as well as the kinetics of the induction were comparable between the suspension culture and soybean seedlings. Using this cell culture, we addressed the question of whether a calcium- and/or calmodulin-mediated step is involved in the signal transduction process between phytochrome and *CAB* expression. We found that W-7, a potent calmodulin antagonist, severely attenuated the induction of *CAB* mRNA by light, whereas W-5, a weak calmodulin antagonist, had little effect. Control experiments demonstrated that W-7 treatment did not block the induction of *hsp-75* by heat shock. The addition of ionomycin, a calcium ionophore, induced a low level of *CAB* mRNA accumulation in the dark which could be further enhanced by light treatment. We propose that calmodulin activation by light is necessary but not sufficient to induce maximal *CAB* expression.

Light plays a critical role in the development of plants. Photoreceptors which perceive different wavelengths of light have been shown to mediate many light-dependent processes (14). Phytochrome is one of the most well-studied photoreceptors and has been implicated to affect physiological functions as diverse as floral induction (23) and chloroplast movement (9). Phytochrome exists in two states: an activated state which absorbs far-red light and a ground state which absorbs red light. In the dark, the former is converted to the latter by thermal decay. However, the two states can be rapidly interconverted by the appropriate light treatment, thus affording a simple assay for phytochrome involvement.

Many plant genes have been shown to respond to light and, more specifically, most of these have been found to be influenced by treatment with far-red light, thus implicating the dependence on activated phytochrome (14). Several light-responsive genes have been shown to be regulated by phytochrome at the transcriptional level (1, 14). The promoters of some of these genes have been characterized with respect to their *cis* element(s) responsible for light responsiveness (1, 14). In addition, two different factors, GT-1 and a G-box-binding protein, have now been reported to bind to light-responsive promoters in vitro (7, 8). In the case of GT-1, site-specific mutation of the critical guanines required for factor binding in vitro can abolish light-responsive expression of a pea *rbcS* promoter in transgenic plants (13). This correlation implicates GT-1 in some process of light-responsive gene expression.

In contrast to the progress in *cis*- and *trans*-acting element analyses, relatively few studies have been done to characterize the signal transduction process for phytochrome. The involvement of calcium in this process was proposed by Roux and colleagues some years ago after demonstration of the ability of phytochrome to modulate calcium flux in plant mitochondrion preparations (21) as well as the phosphorylation of proteins in vitro (3). The role of calcium in mediating phytochrome-regulated processes was also documented in the case of germination of fern spores in the dark (25). However, the biochemical basis of the involvement of calcium, if any, in phytochrome-regulated gene expression has been unexplored because of the lack of any appropriate in vitro system. The use of whole plants to study the biochemical basis of signal transduction is wrought with obvious problems, such as the accessibility of chemical activators and inhibitors and the complex distribution of various cell types that might respond to the treatment differently. Analogous to the animal system, a homogeneous and well-characterized cell line will thus be an invaluable tool in the elucidation of the molecular basis of the signal transduction pathway(s) linked to phytochrome. Unfortunately, the expression of light-regulated genes has been found to be severely attenuated in leaf protoplasts and cell cultures from various plant species (5, 22), and no demonstration of gene regulation by phytochrome in a cell suspension has yet been reported. In addition, most plant cells in culture tend to aggregate into microcalli, thus posing a problem for the even distribution of added chemicals.

In the present work, we report the characterization of a stable, photoautotrophic soybean cell suspension. This cell line can be classified as mesophyll cells by physiological (i.e., photoautotrophy) as well as morphological (i.e., round, chlorophyllous, no severe aggregation) criteria. We show by Northern (RNA) blot analyses that genes encoding the major chlorophyll *a*- and *b*-binding protein (*CAB*) are regulated by phytochrome in this cell line. The expression level is similar to that found in young soybean leaves, and the kinetics of the induction process are also similar. In addition, we show by inhibitor analyses that the activation of calmodulin may be a necessary step in this signal transduction process.

MATERIALS AND METHODS

Growth of SB-P cell cultures and light-dark treatments. SB-P cells were grown in 50 ml of KN1 medium containing

* Corresponding author.
† Present address: Waksman Institute of Molecular Biology and AGBIOTECH Center, Rutgers State University, Piscataway, NJ 08855.

basic Murashige-Skoog salts (Hazleton Biologics), 0.1 mg of thiamine per liter, 1 mg of α-naphthaleneacetic acid per liter, 0.2 mg of kinetin per liter, and 10 g of sucrose per liter (pH 5.7 to 5.9) in 250-ml flasks. The cells were shaken at 120 rpm under continuous light and subcultured every other week by 1/10 dilution. For dark adaptation, the flasks of cell cultures were wrapped with two layers of aluminum foil and shaken as usual for 2 days. For phytochrome treatment, the foil was removed in a dark room and the flasks were irradiated with red and/or far-red light as described previously for experiments with tobacco plants (16). After the light treatment, the flasks were wrapped again with two layers of aluminum foil and returned to the shaker until samples were collected for RNA isolation.

Isolation of RNA from SB-P cells and Northern blot analysis. SB-P cells were collected on P5 filter paper (Fisher Scientific Co.) by vacuum filtration, immediately decanted into liquid nitrogen, and ground to a powder with a mortar and pestle. The powder was thawed in 5 ml of RNA extraction buffer (50 mM of Tris hydrochloride, 300 mM NaCl, 5 mM EDTA, 2% sodium dodecyl sulfate, 2 mM aurintricarboxylic acid, 10 mM mercaptoethanol [pH 8.0]). KCl (3 M; 0.75 ml) was added, and the mixture was incubated on ice for at least 15 min before centrifugation at 7,000 rpm for 10 min in an SS34 rotor (Beckman Instruments, Inc.). LiCl (8 M; 2 ml) was added to the supernatant and, after mixing, RNA was precipitated overnight at 4°C. RNA was collected by centrifugation at 7,000 rpm, and the pellet was suspended in 2 ml of sterile water before extraction with 1 volume of neutralized phenol. RNA in the aqueous phase was precipitated by the addition of 0.2 ml of 3 M sodium acetate (pH 4.6) and 5 ml of ethanol. The pellet was washed with cold 80% (vol/vol) ethanol before suspension in 0.2 to 0.5 ml of sterile water. The RNA concentration was determined by measuring the A_{280}. RNA samples (20 μg each) were dried down before suspension in denaturing buffer containing 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, 50% formamide, 5% formaldehyde, 5% glycerol, and 0.005% bromophenol blue. After being heated for 5 min at 50°C, the samples were loaded onto 1% agarose gels with 0.5% formaldehyde and electrophoresed at 150 V for 1.5 h. After electrophoresis, the RNA was blotted onto nitrocellulose and the blots were hybridized with ^{32}P-labeled DNA probes as described previously (18). The probes were prepared by labeling the isolated cDNA inserts from clones for soybean *CAB* and *hsp75* as described previously (2, 24).

Inhibitor and ionophore treatments. Inhibitors (W-7, W-5, and trifluoroperazine) were purchased from Sigma Chemical Co. and dissolved in dimethyl sulfoxide (DMSO) before use. Ionomycin was obtained from Calbiochem-Behring and also dissolved in DMSO. The chemicals were added to the cell suspension in the dark under a dim green safelight. After the addition, the cells were immediately covered again with two layers of aluminum foil and returned to the shaker for 2 h before further light treatment. No calcium was added with ionomycin, since the cation was already present in the Murashige-Skoog salts.

RESULTS

Quantitative and kinetic characterization of light-regulated *CAB* gene expression in SB-P cell cultures. The photoautotrophic soybean cell culture, SB-P, was first reported in 1983 by Horn et al. (11). This culture was selected from calli of soybeans (*Glycine max* cv. Corsoy) and is able to grow photoautotrophically in an atmosphere of 5% CO_2. Its chlorophyll content is similar to that of soybean leaves on a dry-weight basis, and its growth is inhibited by diuron, a photosynthesis electron transport inhibitor. This cell suspension exhibits light-dependent greening after being cultured for several generations in complete darkness, and a corresponding increase in translatable mRNA for *CAB* was also observed during the greening process (4). In the present work, we first addressed the question of whether the expression of *CAB* is responsive to light in an SB-P cell suspension grown under constant light. We chose a photomixotrophic environment for our culture condition, since it obviates the need for 5% CO_2 in the gas phase. The *CAB* mRNA level in a light-grown culture of the SB-P cells was similar to that in young soybean seedlings (Fig. 1A). Dark adaptation of the SB-P cells led to a reduction in the *CAB* mRNA to a barely detectable level. Titration with total RNA on a Northern blot revealed a 20- to 50-fold difference in the mRNA level between light-grown and dark-adapted SB-P cultures (data

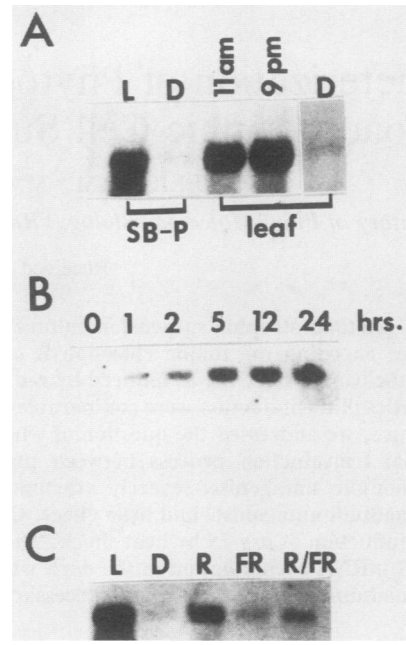

FIG. 1. Characterization of light-regulated *CAB* expression in SB-P cell cultures. (A) Light-grown cells 7 days after subculturing were covered with foil (lane D) or left in the light (lane L). Cells were collected after 2 days, and total RNAs were analyzed by Northern blot analysis as described in Materials and Methods. Soybean seedlings were germinated in vermiculite and grown in a greenhouse for about 2 weeks. The young, expanded leaves were collected either at 11 a.m. or at 9 p.m. RNAs from the leaves were frozen in liquid nitrogen and treated by the same method as that used for the cell suspension. For the dark treatment (lane D), young soybean seedlings were transferred into dark boxes in a darkroom for 2 days before leaves were harvested. (B) The kinetics of light induction of *CAB* mRNA are shown. SB-P cells grown in the light for 1.5 weeks were dark adapted for 2 days. At time zero, the cells were exposed to constant white light. Samples were collected at various times after the shift into light as indicated. (C) Demonstration of phytochrome involvement. Cells were dark adapted for 2 days and then treated with red light (lane R), far-red light (lane FR) or red light followed by far-red light (lane R/FR) as described in Materials and Methods. Cells were returned to darkness after light treatment, and after 16 h total RNA isolated from each sample was analyzed by Northern blot analysis.

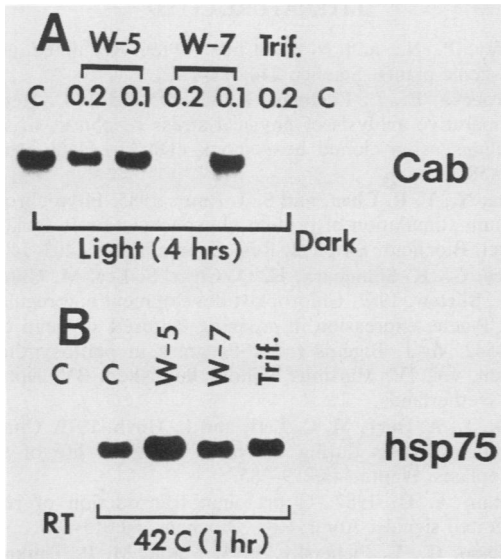

FIG. 2. Effects of calmodulin inhibitors on *CAB* induction by light. (A) Effects of W-7, W-5, and trifluoroperazine (Trif.) on light-induced *CAB* expression. SB-P cells were dark adapted for 2 days, and the inhibitors (lanes W-5, W-7, and Trif.) or DMSO alone (lane C) was added to the cultures in a darkroom under a dim green safelight. Cells were incubated in the dark for 2 h before a shift to constant white light. Cells were collected for RNA preparation after 4 h in the light. As a control (lane C, Dark), one set of cells was left in darkness throughout. Numbers above lanes represent inhibitor concentrations (millimolar). (B) Effect of the inhibitors on heat induction of *hsp75*. Cells were treated in the same way as described for the light experiment. However, instead of being exposed to constant white light, the cells in the dark were immersed in a 42°C water bath for 1 h before RNA isolation. A sample left at room temperature (lane C, RT) was included as a control. The slightly more intense signal on the lane containing the W-5-treated sample in the heat shock experiment is an artifact of RNA loading. It does not represent a reproducible difference in separate experiments.

not shown). After exposure of a dark-adapted SB-P culture to constant light, the activation of *CAB* expression was observed in 1 h and the mRNA level reached a steady state between 5 and 12 h after the transfer into light (Fig. 1B). This result is similar to the induction kinetics of *CAB* expression in dark-grown soybean seedlings (15) and in 2-day dark-adapted soybean seedlings (data not shown).

Since *CAB* from several plant species had been reported (17, 20) to be regulated by an endogenous circadian clock, we collected the soybean leaves in the morning and evening to ensure against underestimating the expression level in leaves. However, we did not find any significant difference in *CAB* mRNA levels between morning and evening, unlike the observation with species such as wheat (17) and tobacco (20).

We next examined whether the light-dependent expression of *CAB* in the SB-P culture is mediated by phytochrome. After dark adaptation for 2 days, a 5-min treatment with red light was sufficient to activate *CAB* expression to about 50% the level found in light-grown cells (Fig. 1C). Far-red light (15 min) given immediately after red light effectively attenuated *CAB* expression to a similar level as far-red light alone. The ability of far-red light to induce low-level *CAB* expression is characteristic of the very-low-fluence response known to be associated with the induction of *CAB* in other systems (12). These results strongly suggest that the regulation of *CAB* in the SB-P culture is very similar, if not identical, to that in soybean leaves.

Requirement for calmodulin in light activation of *CAB* in SB-P cell cultures. Calcium and, more specifically, calmodulin, have been implicated in the signal transduction process linked to phytochrome (3, 9, 25). We wanted to examine this possibility in the SB-P system, since the accessibility problem is minimized in the cell suspension system. We tested the effects of a potent calmodulin inhibitor, W-7, on the induction of *CAB* by light. As controls, we also tested the relatively inactive analog W-5 (10) and examined the effects of these inhibitors on heat induction of soybean *hsp75*, which is responsive to elevated temperatures (2). *CAB* induction by light was severely attenuated after treatment with 0.2 mM W-7 (Fig. 2). Under similar conditions, induction of *hsp75* by heat treatment was not affected. At the same concentration, W-5 had much less of an effect, although at higher concentrations (i.e., 0.5 mM), it began to have more severe inhibitory effects (data not shown). Since the accumulation of the heat-inducible transcript was not affected, these data suggest that treatment with W-7 does not cause a general inhibition of transcription or an acceleration of mRNA turnover. The quantitative difference between the potencies of W-7 and W-5 indicates that the target of both compounds is likely to be calmodulin (10). Consistent with this conclusion is the observation that another calmodulin antagonist, trifluoroperazine, also inhibited *CAB* induction by light in this system (Fig. 2).

If the induction of *CAB* by phytochrome requires only the activation of calmodulin, then it should be possible to induce the expression of *CAB* in the dark by increasing the intracellular calcium concentration with a calcium-specific ionophore. Treatment of the SB-P cells with the calcium-specific ionophore ionomycin caused a rapid accumulation of a low level of *CAB* mRNA (i.e., after 1 h) (Fig. 3). However, the maximal level of this induction never exceeded more than 10% of that found in light-treated SB-P cells. Since the presence of light can activate *CAB* normally in the presence of ionomycin, the addition of the ionophore probably did not cause any pleiotropic effects on the phytochrome signal transduction chain. We consider the slight induction of *CAB* to be specific, since *hsp75* was not induced by the same

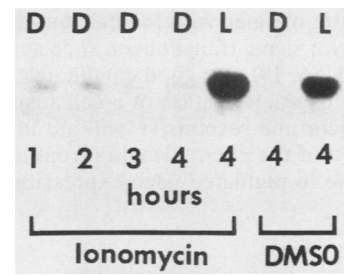

FIG. 3. Effect of ionomycin on *CAB* expression in dark-adapted SB-P Cells. Eight flasks (50 ml each) of SB-P cells were dark adapted for 2 days. Each of the eight flasks was divided into 25-ml portions in the dark under a dim green safelight. Ionomycin (10 μM) in DMSO or an equal volume of DMSO was added to the cells in the dark before the cells were covered again with aluminum foil. The cell portions were exposed to the dim green safelight for about the same lengths of time. Flasks from the control and the ionomycin-treated cells were immediately shifted to constant white light for 4 h. For the ionomycin-treated flasks, cells were collected 1, 2, 3, and 4 h after the treatment. For the DMSO control flasks, only the 4-h cells were collected. Northern blot analysis with a *CAB* cDNA probe is shown. D, Dark; L, light.

ionomycin treatment (data not shown). Thus, these results suggest that activated calmodulin is required but is insufficient on its own to induce maximal *CAB* expression.

DISCUSSION

In the present work, we explored the use of a photoautotrophic cell suspension from soybeans as a model system for studying the signal transduction events between phytochrome and light-responsive gene expression. As a first step, we demonstrated by Northern blot analysis that the expression of *CAB* in cultures grown under photomixotrophic conditions is critically dependent on light. More specifically, we showed that phytochrome can activate *CAB* in these cells after dark adaptation. Quantitatively as well as kinetically, the regulation of *CAB* in these cells is very similar to that found in soybean leaves. Since these studies can be carried out at ambient CO_2 concentrations under photomixotrophic conditions, this cell culture thus provides an easily manipulatable system without any complicated experimental set-up. This cell line is also quite homogeneous morphologically and so should be ideal for biochemical as well as molecular analyses. Since phytochrome is known to be capable of regulating calcium fluxes in vitro (21), we decided to examine the dependence of light induction of *CAB* on calmodulin by using characterized inhibitors. The results were consistent with a model in which *CAB* activation by light requires calmodulin activation. In contrast, heat induction of *hsp75* was not affected by the inhibitors under nearly identical conditions, demonstrating that the expression of this inducible gene is not likely to be mediated by calmodulin in this system.

Studies with calcium flux artificially induced by treatment with a calcium ionophore suggested that phytochrome induction of *CAB* is not mediated simply by an increase in intracellular calcium. Thus, although our study with calmodulin antagonists clearly implicated the requirement for a calcium and/or calmodulin step in the phytochrome signal transduction process leading to *CAB* expression, another critical component(s) for this light-responsive pathway remains to be defined. Since our inhibitor study showed that W-7 can completely block the activation of *CAB* by light, this other necessary second messenger(s) is likely to act in a highly synergistic manner with the calcium-dependent step. Other well-known signal transducers, such as G proteins and protein kinase C (6, 19), are good candidates at present. We expect that the characterization of a cell suspension system which is phytochrome responsive will aid in the molecular characterization of the events leading from light perception by phytochrome to regulated gene expression.

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