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Pr-Specific Phytochrome Phosphorylation in Vitro by a Protein Kinase Present in Anti-Phytochrome Maize Immunoprecipitates[1]

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Protein kinase activity has repeatedly been found to co-purify with the plant photoreceptor phytochrome, suggesting that light signals received by phytochrome may be transduced or modulated through protein phosphorylation. In this study immunoprecipitation techniques were used to characterize protein kinase activity associated with phytochrome from maize (*Zea mays* L.). A protein kinase that specifically phosphorylated phytochrome was present in washed anti-phytochrome immunoprecipitates of etiolated coleoptile proteins. No other substrate tested was phosphorylated by this kinase. Adding salts or detergents to disrupt low-affinity protein interactions reduced background phosphorylation in immunoprecipitates without affecting phytochrome phosphorylation, indicating that the protein kinase catalytic activity is either intrinsic to the phytochrome molecule or associated with it by high-affinity interactions. Red irradiation (of coleoptiles or extracts) sufficient to approach photoconversion saturation reduced phosphorylation of immunoprecipitated phytochrome. Subsequent far-red irradiation reversed the red-light effect. Phytochrome phosphorylation was stimulated about 10-fold by a co-immunoprecipitated factor. The stimulatory factor was highest in immunoprecipitates when Mg^{2+} was present in immunoprecipitation reactions but remained in the supernatant in the absence of Mg^{2+}. These observations provide strong support for the hypothesis that phytochrome-associated protein kinase modulates light responses in vivo. Since only phytochrome was found to be phosphorylated, the co-immunoprecipitated protein kinase may function to regulate receptor activity.

The plant photoreceptor phytochrome mediates light regulation of diverse phenomena such as seed germination, seedling morphogenesis, chloroplast development, root gravitropism, and flower initiation. Phytochrome can exist in two photointerconvertible forms, one (Pr) that absorbs primarily red light, and the other (Pfr) that has a main absorption peak in the far-red region of the spectrum. Pfr is considered to be the active form, since conversion of only a small proportion of phytochrome to this form is sufficient to trigger many biological responses. Very-low-fluence phytochrome-mediated biological responses of etiolated oat seedlings are not completely reversible by far-red light and have a red-light threshold at approximately 1×10^{-10} mol m^{-2} (corresponding to 0.01% Pfr), saturating at 1×10^{-7} mol m^{-2} (0.4% Pfr) (Mandoli and Briggs, 1981). Low-fluence biological responses and phytochrome photoconversion are both far-red reversible; in maize (*Zea mays* L.) and oat coleoptiles both approach saturation at approximately 1×10^{-3} mol m^{-2} (87% Pfr) (Briggs and Chon, 1966; Mandoli and Briggs, 1981).

Red or far-red light activation of the phytochrome chromophore induces conformational changes in the phytochrome apoprotein that have been well documented (Shimazaki et al., 1986; Wong et al., 1986). However, little is known about the events that occur immediately after phytochrome photostimulation in signal-transduction pathways involving this light receptor (reviewed by Kronenberg and Kendrick, 1986). Later events, including binding of transcription factors to light-regulated promoter elements, have recently been elucidated (Datta and Cashmore, 1989; Dehesh et al., 1990, Sarokin and Chua, 1992). Activation of heterotrimeric GTP-binding (G-) proteins or cytosolic calcium/calmodulin stimulates transcription of certain light-regulated genes and is hypothesized to be part of the primary light-transduction pathway (Romero et al., 1991; Neuhaus et al., 1993; Romero and Lam, 1993). However, the maximum transcriptional activation by G-protein modulators is only 25% of that caused by light (Romero and Lam, 1993), indicating that G-protein-independent steps may also transduce light signals. Red light induces a transient increase in cytosolic calcium (Shacklock et al., 1992) and activates calcium- and calmodulin-dependent signal-transduction pathways (Datta et al., 1985; Lew et al., 1990; Fallon et al., 1993).

Two types of evidence suggest that protein phosphorylation may be involved in transduction of light signals by phytochrome. First, protein kinase activity has been found to be associated with purified phytochrome by several research groups (Grimm et al., 1989; Kim et al., 1989; Wong et al., 1989), although it is unclear if these groups were examining the same kinase activity. Since kinase activity could not be found apart from phytochrome, the activity appears to be either intrinsic to the phytochrome polypeptide or residing in another polypeptide that is tightly bound to phytochrome and that requires phytochrome for activity (Kim et al., 1989; Wong et al., 1989). However, in other studies phytochrome without protein kinase activity could be separated from phytochrome with protein kinase activity, suggesting that the

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Abbreviation: PAPK, phytochrome-associated protein kinase.

activity is not intrinsic to the phytochrome molecule (Grimm et al., 1989; Kim et al., 1989). It is difficult in such studies to completely eliminate the possibility of loss of intrinsic protein kinase activity by denaturation during separation procedures. The question of whether or not phytochrome is a protein kinase has not been resolved. The view that protein kinase activity is intrinsic to the phytochrome molecule is supported by specific binding of ATP analogs to phytochrome (Wong and Lagarias, 1989). However, angiosperm phytochrome does not exhibit any of the conserved structural features characteristic of eukaryotic receptors that have intrinsic protein kinase activity (Hanks et al., 1988), suggesting that nucleotide binding may be required for some phytochrome catalytic function other than protein phosphorylation.

The far-red reversibility of red-light effects on protein phosphorylation in vivo further indicate that protein phosphorylation is involved in phytochrome responses (Datta et al., 1985; Otto and Schäfer, 1988; Fallon et al., 1993). Red light stimulates rapid phosphorylation or dephosphorylation of specific proteins in etiolated oat coleoptile tips (Otto and Schäfer, 1988) and also stimulates calcium-dependent phosphorylation of proteins in isolated pea nuclei (Datta et al., 1985) and wheat protoplasts (Fallon et al., 1993). The phosphorylation-dependent binding of transcription factors to light-regulated promoter elements (Datta and Cashmore, 1989; Sarokin and Chua, 1992) is additional evidence that phytochrome responses are transduced by pathways employing reversible protein phosphorylation.

Co-immunoprecipitation of a protein kinase catalytic region with an associated protein is a technique that has been essential in elucidation of molecular details of the regulation of other protein kinases (p34 protein kinase regulation by cyclins in animals and yeast) (Draetta et al., 1989; Wittenberg et al., 1990). Thus, immunoprecipitation might likewise provide information concerning PAPK. Based on the co-purification studies cited above, we hypothesized that phytochrome immunoprecipitates would contain protein kinase activity intrinsic to phytochrome or residing in an associated peptide. After verifying this hypothesis, the following biochemical properties of PAPK were studied: substrate specificity, light regulation, and effect of phytochrome-binding cellular components.

MATERIALS AND METHODS

Light

All steps, including tissue harvesting, protein extraction, immunoprecipitation, and phosphorylation assays, described below were conducted in darkness or very dim green light (using a green filter with a band pass of 480–560 nm, peak at 520 nm; fluence rate approximately 1×10^{-9} mol m^{-2} s^{-1} as measured with a Li-Cor model LI-189 light meter), unless otherwise specified. Red-light source was a slide projector with light passed through a 640 ± 10 nm band-pass filter. Far-red light was obtained using an incandescent bulb with light passed through a wide band-pass filter with a peak emission at 750 nm and no detectable emission below 700 nm. These light sources provided 1.7×10^{-5} mol m^{-2} s^{-1} (red) and 5×10^{-4} mol m^{-2} s^{-1} (far red). White light (2×10^{-5} mol m^{-2} s^{-1}) was provided by a cool-white fluorescent bulb. Blue light (1×10^{-7} m^{-2} s^{-1}) was from an incandescent bulb covered with a filter with peak transmission at 410 nm and no emission detectable below 350 nm or above 470 nm. IR light (>800 nm) was obtained from an incandescent bulb covered with a Kodak 87C Wratten gelatin filter and was visualized using a Find-R-scope (FJW Industries, Mt. Prospect, IL) that converted IR light into visible green light.

Tissue Extraction

Maize (*Zea mays* L. var Merit) seeds were germinated and seedlings were grown in darkness for 4 d (Feldman et al., 1988), then coleoptiles were excised and used immediately or frozen at −80°C. Soluble proteins were extracted by homogenization with a Brinkmann Polytron (1 min, medium power) in 5 volumes of extraction buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM DTT, 2 mM mercaptoethanol) to which polyvinylpolypyrrolidone (1%, w/v) had been added.

The following proteinase inhibitors were added freshly to the extraction buffer and to all other buffers used in immunoprecipitation and phosphorylation assays: PMSF to 1 mM; aprotinin and leupeptin to 1 μg/mL; and antipain to 5 μg/mL. Tissue homogenates were filtered through a 50-μm Nitex nylon monofilament mesh, then centrifuged for 15 min at 13,000*g*, 4°C, to remove insoluble material. One-milliliter aliquots of extract were then immediately immunoprecipitated or stored at −80°C prior to immunoprecipitation. Gel-filtered coleoptile proteins were prepared as follows: protein extract (above) was concentrated by precipitation with ammonium sulfate to 85% saturation and resuspended at 10 mg/mL in extraction buffer, then 10 mL of the extract was desalted in the same buffer on a 5-cm diameter × 20-cm-long column of Sephadex G-200 (40–120 μm). Fractions containing more than 0.2 mg/mL protein were pooled, giving a final protein concentration of 2 mg/mL.

Immunoprecipitation

A polyclonal rabbit antiserum (No. 4021, a gift from Peter Quail) raised against highly purified oat phytochrome (prepared as Pfr by the method of Vierstra and Quail, 1983) was used in these experiments. Highly purified phytochrome prepared as Pfr does not exhibit protein kinase activity (Grimm et al., 1989; Wong et al., 1989), and this antiserum would thus not contain antibodies directed against putative co-purifying protein kinase peptides. On immunoblots this antiserum reacts only with a 127-kD protein that is abundant in etiolated coleoptiles but is not detectable in extracts of coleoptiles illuminated for 24 h. We immobilized antibody molecules on protein A beads prior to reacting with phytochrome (rather than collecting immunoprecipitates with protein A beads, which is the usual procedure). This was done to minimize polyvalency of antibody binding to phytochrome (which was expected to interfere with phytochrome photoconversion and other biochemical activities), since steric constraints would reduce binding of additional immobilized antibody molecules to IgG-bound phytochrome.

To immobilize IgG, 3 μL of anti-phytochrome serum was

mixed with 20 μL of protein A Trisacryl beads (Pierce) in 500 μL of PBS (with proteinase inhibitors; see above), and gently rocked for 30 min at 4°C. Unbound serum proteins were removed by two 1-mL washes in the same buffer. Beads were pelleted by microcentrifugation for 10 s prior to removing washes. The immobilized antibody, or control nonimmune IgG from another rabbit, were then reacted in darkness with 1 mL of coleoptile extract (about 2 mg of protein) by gently shaking at 4°C for 1.5 to 2 h. Unless otherwise specified, immune complexes thus formed were washed three times by resuspension in 1 mL of RIPA buffer (extraction buffer with 150 mM NaCl, 1% [v/v] Triton X-100, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS), once in RIPA buffer without sodium deoxycholate or SDS, and once in protein kinase assay buffer (without ATP), and centrifuged as above. Extraction buffer was used for washes preceding irradiation (and comparison unirradiated samples) to prevent possible impairment of photoconversion resulting from detergent denaturation.

In Vitro Protein Phosphorylation Assays

Ten microliters of immune complexes immobilized on protein A beads, suspended in 10 μL of protein phosphorylation buffer (without ATP), were pipetted into individual wells of a 96-well U-bottom micro-assay plate on ice. Ten microliters of protein phosphorylation buffer was added [25 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2.5 mM NaF, 5 mM DTT, 10 nM protein phosphatase inhibitor microcystin-LR, ADP scavenging system (100 units/mL creatine phosphokinase with 4 mM phosphocreatine) and 20 nM ATP (2 μCi [γ-^{32}P]ATP, 5000 Ci/mmol, per 20-μL reaction)]. Five hundred nanomolar ATP (200 Ci ^{32}P/mmol) was used in assays to test phosphorylation of added substrates. Protein kinase substrates were purchased in dephosphorylated form from Sigma, and 20 μg was added to assays at the same time as ATP. Assays were incubated at 25°C for 30 min with rocking sufficient to circulate solutions surrounding immunoprecipitates, then phosphorylation was terminated on ice by adding (in the same sample sequence) 10 μL of 3× SDS-PAGE buffer.

Electrophoresis

Samples were denatured by heating for 3 min at 90°C, then mixed by pipetting. Gravity supernatants were pipetted with a sequencing tip and electrophoresed on mini-gels (Laemmli, 1970), removing [^{32}P]ATP by excising the ion front from the bottom of the gel. Gels were then stained with Coomassie blue in fixer, dried, and autoradiographed for 1 to 2 d without screens. The amount of Coomassie blue-stained phytochrome in individual bands was visually assessed, and xerographic records were made of dried stained ^{32}P gels. Where indicated, phytochrome bands (clearly visible by Coomassie stain) were excised with a razor blade and cpm was determined by scintillation counting.

RESULTS

Phytochrome Is Specifically Phosphorylated by a Kinase through High-Affinity Interactions

PAPK was studied in immunoprecipitates formed by incubating protein A-immobilized antibodies with maize coleoptile extracts. Protein kinase activity in washed immunoprecipitates was detected by in vitro labeling immobilized immune complexes with [γ-^{32}P]ATP, then electrophoretically separating and autoradiographing proteins. An approximately 127-kD protein was phosphorylated in vitro in washed immunoprecipitates formed with a polyclonal anti-phytochrome rabbit serum, but not in controls formed with nonimmune sera (Fig. 1A). This corresponded with the position of phytochrome as determined by western blotting of immune complexes (data not shown). No peptides besides phytochrome were observed on Coomassie blue-stained gels to be specifically associated with anti-phytochrome versus nonimmune precipitates (data not shown), although immunoglobulin peptides or protein A could have obscured comigrating proteins. Likewise, no ^{32}P-labeled proteins besides phytochrome were more abundant in anti-phytochrome than in nonimmune precipitates. Phosphoryl labeling of immunoprecipitated phytochrome increased linearly over a 30-min period (Fig. 1B) and did not occur in the absence of Mg^{2+} (data not shown).

Detergents and salts were added to immunoprecipitate wash buffer to test if the protein kinase was associated with phytochrome immunoprecipitates by disruptible low-affinity interactions or high-affinity biochemical interactions, which are stable under these conditions (Draetta et al., 1989; Wittenberg et al., 1990). Detergent-containing washes did not affect phytochrome phosphorylation but they did reduce background phosphorylation of all proteins besides phytochrome in the immune complexes, compared with detergent-free washes (Fig. 2), indicating that the protein kinase is integral or is associated with phytochrome by high-affinity interactions.

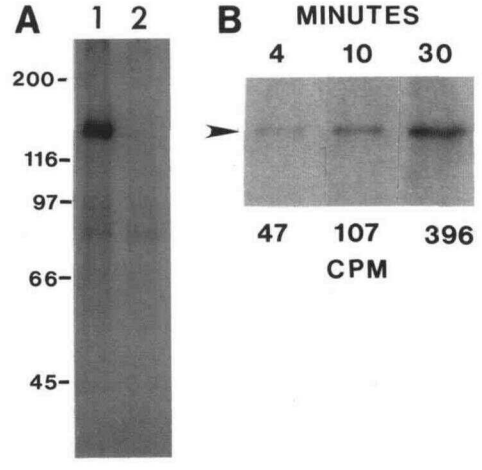

Figure 1. Immunoprecipitated phytochrome is phosphorylated in vitro. A, Comparison with nonimmune control. Protein extract from etiolated maize coleoptile was incubated with immobilized anti-phytochrome (lane 1) or nonimmune (lane 2) serum. Washed immunoprecipitates were phosphorylated in vitro with [^{32}P]ATP, then proteins were electrophoresed and autoradiographed. Numbers at left denote mol wts of marker proteins at those positions. B, As in A, anti-phytochrome immunoprecipitates were phosphorylated for different times. ^{32}P activity (cpm) of the excised phytochrome band is shown below each gel lane. Arrowhead at left shows position of phosphorylated phytochrome.

To determine whether PAPK specifically phosphorylated phytochrome or had other in vitro substrates, various substrates commonly used to assay multifunctional protein kinases were added to phosphorylation reactions containing immunoprecipitates. Histone type IIA, Arg-rich histone fraction, Lys-rich histone fraction, myelin basic protein, protamine, or casein were not phosphorylated in vitro when added to phytochrome immunoprecipitates from etiolated coleoptiles or coleoptiles irradiated with white light (1.2×10^{-3} mol m^{-2}) immediately prior to extraction, compared with control preimmune precipitates (Fig. 3A, and other data not shown). Added bovine cAMP-dependent protein kinase or maize extracts containing endogenous calcium-dependent protein kinase phosphorylated these substrates in control reactions containing immunoprecipitates, but did not increase phytochrome phosphorylation. No substrates of PAPK were detectable when maize coleoptile proteins were added to phosphorylation reactions, but addition of maize proteins reduced phytochrome phosphorylation (Fig. 3B). Compounds known to stimulate other protein kinases were tested for effects on phosphorylation of immunoprecipitated phytochrome or added histone type IIA (a substrate of the protein kinase copurified with phytochrome by Wong et al., 1986). Polycations such as polylysine and histones did not affect phosphorylation of immunoprecipitated phytochrome when added to phosphorylation assays. The second messengers cAMP, cGMP, and calcium were also not stimulatory (Table I).

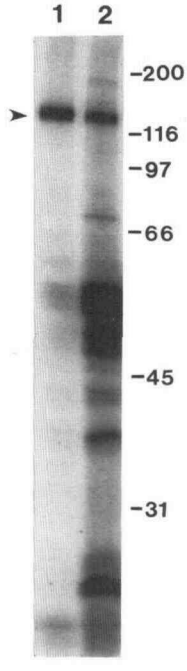

Figure 2. The protein kinase that phosphorylates phytochrome is bound to immunoprecipitates by salt- and detergent-stable interactions. Anti-phytochrome precipitates (from etiolated coleoptiles) were washed four times in protein extraction buffer to which had been added 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl (lane 1), or were washed four times in protein extraction buffer (lane 2). Washed immunoprecipitates were phosphorylated in vitro with [^{32}P]ATP, then proteins were electrophoresed and autoradiographed. Numbers at right denote mol wts of marker proteins at those positions. Arrowhead at left shows phosphorylated phytochrome.

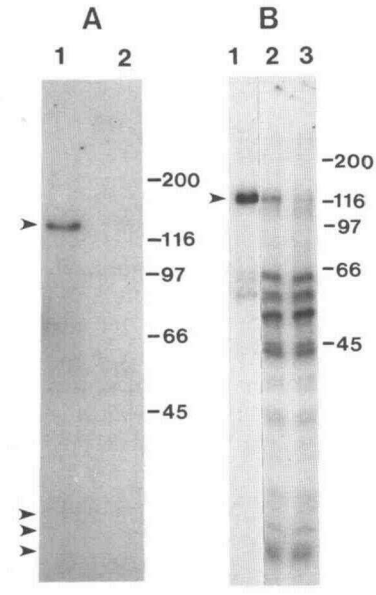

Figure 3. Substrate phosphorylation by the protein kinase present in anti-phytochrome precipitates is highly specific in vitro. A, Histone IIA (1 $\mu g/\mu L$) is not phosphorylated in reactions containing phytochrome immunoprecipitate from unirradiated coleoptiles (lane 1) or tissue irradiated for 1 min with white light (lane 2) or nonimmune precipitates from either tissue (not shown). Lower left arrowheads show position of histone peptides; upper arrowheads shows phosphorylated phytochrome. B, In vitro phosphorylation of any coleoptile proteins is not increased by addition of phytochrome immunoprecipitates. Phytochrome was immunoprecipitated from unirradiated tissue, then phosphorylated in vitro (lane 1) or 20 μg of gel-filtered coleoptile proteins was added to the immunoprecipitate before phosphorylation (lane 2). Lane 3 shows 20 μg of coleoptile proteins phosphorylated without phytochrome immunoprecipitate.

PAPK Phosphorylates Immunoprecipitated Pr but Not Pfr

Irradiation of whole coleoptiles or plant extracts with a fluence of red light sufficient to approach photoconversion saturation (1.0×10^{-3} mol m^{-2}) prior to immunoprecipitation greatly reduced subsequent phytochrome phosphorylation in vitro compared with unirradiated controls (Fig. 4A). Since Pr still accounts for 13% of phytochrome after saturating red irradiation, labeling of red-irradiated phytochrome (11–14% of safelight controls, Figs. 4A and 5) could be completely accounted for by phosphorylation of residual Pr, with no phosphorylation of Pfr. White light (1.2×10^{-3} mol m^{-2}) had an effect similar to that of red light (Fig. 4A). A lower fluence of red light (1.7×10^{-5} mol m^{-2}), sufficient to saturate very-low-fluence biological responses but not to photoconvert a large proportion of Pr to Pfr (Briggs and Chon, 1966; Mandoli and Briggs, 1981; Feldman and Briggs, 1987), did not have significant effect on phosphorylation of subsequently immunoprecipitated phytochrome compared with safelight controls (Fig. 4A). Irradiation of coleoptiles with blue light (6 ×

Table I. *Potential protein phosphorylation regulators that had no effect on phytochrome or histone IIA phosphorylation by the protein kinase present in immunoprecipitates*

Regulator	Concentration(s) Tested
Calcium	1 mM (or 2 mM EGTA)
cAMP	100 nM, 1 μM, 10 μM
cGMP	100 nM, 1 μM, 10 μM
Polylysine	0.01, 0.1, or 1 mg/mL
DTT	10 mM (or omitted)
Glc-6-P	100 μM, 1 mM
Microcystin	10 nM (or omitted)

10^{-5} mol m^{-2}) just prior to tissue extraction did not affect subsequent phosphorylation of immunoprecipitated phytochrome (data not shown). Amounts of immunoprecipitated phytochrome detected by Coomassie blue staining were similar for the various light treatments (Fig. 4A). The effect of photoconversion-saturating red or white irradiation was fully reversible by subsequent far-red irradiation (Fig. 4, B and C). In vitro irradiation of protein extracts affected phosphorylation of subsequently immunoprecipitated phytochrome similar to in vivo irradiation (Fig. 4, B and C).

Irradiation from the green safelight would generate amounts of Pfr sufficient to trigger very-low-fluence biological responses and thus obscure very-low-fluence light effects on activity of PAPK. To test if small amounts of Pfr affected Pr phosphorylation in immunoprecipitates, phytochrome was immunoprecipitated from coleoptiles either kept in darkness or irradiated with far-red light (to saturate very-low-fluence responses but only convert a small fraction of phytochrome to Pfr), then phosphorylated in vitro without further phytochrome photoconversion (for visualization, samples were irradiated only with IR light, which is not absorbed by either form of phytochrome). The presence or absence of a small amount of Pfr did not affect phytochrome phosphorylation by the associated protein kinase (Fig. 4D).

Reduction of phytochrome phosphorylation by red irradiation could have been caused by preferential phosphorylation of Pr rather than Pfr, or by red-light-induced dissociation of a protein kinase catalytic peptide or other factor required for activity. Therefore, we investigated whether the protein kinase remained associated with immunoprecipitates following saturating red irradiation. High-fluence irradiation reduced phytochrome phosphorylation, whether of lysate, unwashed immunoprecipitate, or washed immunoprecipitate (Fig. 5), indicating that photoconversion of the immunoprecipitated phytochrome was efficient. Far-red irradiation reversed the effect of red irradiation at all these times, even when immunoprecipitates were washed between irradiations (Fig. 5).

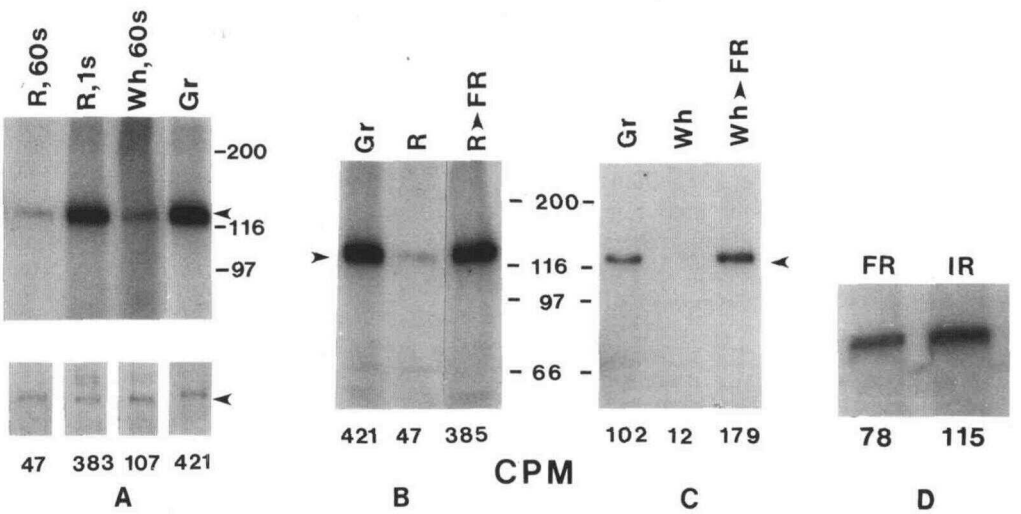

Figure 4. Phosphorylation of immunoprecipitated phytochrome is light regulated. A, Effect of white (Wh) or red (R) irradiation of excised coleoptiles on in vitro phosphorylation of subsequently extracted and immunoprecipitated phytochrome. An autoradiograph of electrophoretically separated proteins is shown. Coleoptiles were irradiated for 1 to 60 s with red (1.7×10^{-5} mol m^{-2} s^{-1}) or white light (2×10^{-5} mol m^{-2} s^{-1}). Controls (Gr) were exposed only to the green safelight. Below is shown the region of the Coomassie blue-stained gel lane containing phytochrome. ^{32}P activity (cpm) of the excised phytochrome band is shown at bottom. Numbers at right denote mol wts of marker proteins at those positions. Arrowhead at right shows phosphorylated phytochrome. B, Reversal of red-light effect in vivo by far-red light. As in A, except 60 s of far-red light (FR, 5×10^{-4} mol m^{-2} s^{-1}) immediately followed 60 s of red (R) irradiation. Green safelight control (Gr) is shown for comparison. The amount of immunoprecipitated phytochrome visible on Coomassie blue-stained gels was similar for the various treatments, but is not shown. C, Reversal of white-light effect in vitro by far-red light. As in A except protein extracts (rather than intact tissues) were irradiated prior to immunoprecipitation. Far-red light (FR, 60 s, 5×10^{-4} mol m^{-2} s^{-1}) immediately followed 60 s of white (Wh) irradiation. ^{32}P activity (cpm) of the excised phytochrome band is shown below each gel lane. The amount of immunoprecipitated phytochrome visible on Coomassie blue-stained gels was similar for the various treatments, but is not shown. A similar experiment with red light had the same result. D, Effect of FR irradiation. As in A, except that all steps were conducted without green irradiation, with only IR irradiation of controls or 60 s of far-red (FR) irradiation of coleoptiles.

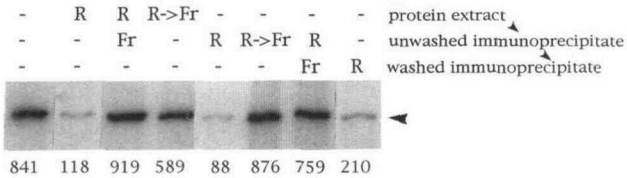

Figure 5. Red-irradiation effect is reversible by far-red light after immunoprecipitation and washing. Protein extracts, unwashed anti-phytochrome immunoprecipitates, or washed immunoprecipitates were irradiated for 60 s with red (R), far-red (Fr), or red light immediately followed by far-red light (R→Fr) as indicated above gel lanes. Washed immunoprecipitates were phosphorylated in vitro with [^{32}P]ATP, then proteins were electrophoresed and autoradiographed. ^{32}P activity (cpm) of the excised phytochrome band is shown below each gel lane. Arrowhead at right shows position of phosphorylated phytochrome. The amount of immunoprecipitated phytochrome visible on Coomassie blue-stained gels was similar for the various treatments, but is not shown.

Thus, the protein kinase remained associated with immunoprecipitated phytochrome, whether the phytochrome was mostly Pr or Pfr.

If only Pr rather than Pfr were phosphorylated by the protein kinase in immunoprecipitates, Pr-specific antibodies might prevent phytochrome phosphorylation by binding at or near the phosphorylation site. Therefore, extracts from etiolated coleoptiles were immunoprecipitated with a monoclonal antibody (1.9B5A, Daniels and Quail, 1984), which binds an epitope located within 6 kD of the N terminus of phytochrome and is preferentially exposed in the Pr form. Phytochrome immunoprecipitated by this antibody was not detectably phosphorylated in vitro (Fig. 6), although the quantity was similar to that in polyclonal immunoprecipitates, as indicated by Coomassie blue staining of the gel.

Mg^{2+}-Dependent Processes Affect the Phosphorylation State of Phytochrome

Mg^{2+}-dependent processes, such as protein phosphorylation and dephosphorylation, or other reactions involving nucleotide binding could affect the phosphorylation state of immunoprecipitated phytochrome, association of other proteins, or activation of co-immunoprecipitated peptides. Therefore, we determined the effect of Mg^{2+} (in protein extracts from which phytochrome was immunoprecipitated) on PAPK. The presence of Mg^{2+} during immunoprecipitation increased subsequent in vitro phosphorylation of phytochrome (Fig. 7A). High-fluence but not low-fluence red irradiation reduced phytochrome phosphorylation when immunoprecipitation took place in the presence of Mg^{2+}, as in previous experiments where phytochrome was immunoprecipitated from an EDTA extraction buffer (Fig. 7A). Phosphorylation of phytochrome immunoprecipitated from an EDTA-containing coleoptile extract was stimulated (to the same level as immunoprecipitation in Mg^{2+}-containing extract) by subsequently reincubating immune complexes in the immunoprecipitation supernatant in the presence of Mg^{2+} (Fig. 7B, lanes 2 and 4). If phytochrome immunoprecipitates had already been incubated in the extract in the presence of Mg^{2+}, the stimulation was far less (Fig. 7B, lanes 2 and 3). Thus, a factor strongly stimulating the phytochrome kinase (or the kinase itself) had co-immunoprecipitated with the phytochrome.

DISCUSSION

Phosphorylation of purified phytochrome in vitro can be used as a probe of its light-induced conformational state, since some purified mammalian protein kinases (cGMP-dependent protein kinase) phosphorylate only the Pr form of phytochrome (Wong et al., 1986). The site(s) phosphorylated by these kinases are located within a conserved N-terminal region exposed in Pr but inaccessible in Pfr (McMichael and Lagarias, 1990). This region of the phytochrome polypeptide has been shown to be necessary for full activity in transgenic plants (Cherry et al., 1992). In this study a protein kinase associated with immunoprecipitated phytochrome was quantitatively affected by light-induced conformational changes that occurred in vivo. Pr was phosphorylated with little or no phosphorylation of Pfr (Figs. 4 and 5) and photoconversion in vitro had the same effect on subsequent phytochrome phosphorylation as illumination in vivo. Thus, the PAPK can distinguish between light-induced conformational states in a manner similar to second messenger-regulated protein kinases.

The lack of phosphorylation of Pfr is not due to absence of protein kinase peptide in Pfr immunoprecipitates since Pr-phosphorylating activity remained associated with washed Pfr immunoprecipitates (Fig. 5). However, Pr was not phosphorylated when it was immunoprecipitated by a monoclonal antibody that reacts with an N-terminal region accessible only in the Pr form (Daniels and Quail, 1984; Fig. 6), possibly due to phosphorylation site blockage by the antibody. Since it is already known that at least two N-terminal phosphorylatable sites (and none in other regions) are exposed in Pr but not Pfr, these data together indicate that the immunoprecipitated protein kinase probably phosphorylates site(s) in the Pr-exposed N-terminal region, although proof of this will require phosphopeptide mapping.

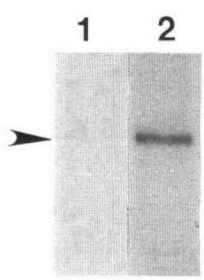

Figure 6. Phytochrome immunoprecipitated with monoclonal antiserum recognizing a N-terminal Pr-specific epitope is not phosphorylated in vitro. Phytochrome from etiolated coleoptiles was immunoprecipitated with monoclonal antibody 1.9 B5A (lane 1) or polyclonal antisera (lane 2), then immunoprecipitates were phosphorylated in vitro. An autoradiograph of electrophoresed proteins is shown. Arrowhead shows position of the Coomassie blue-stained phytochrome band. The amount of phytochrome detected by Coomassie blue staining was similar for the two antisera.

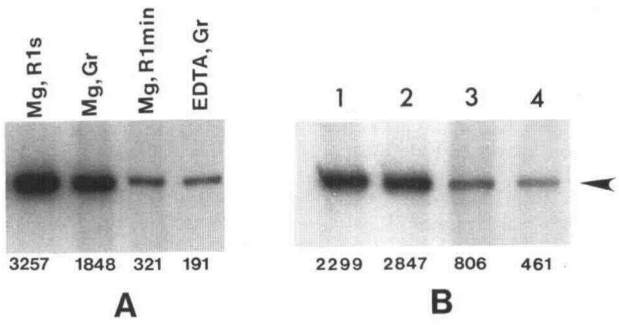

Figure 7. Phytochrome phosphorylation is stimulated by a co-immunoprecipitated factor whose binding to phytochrome is dependent on the presence of Mg^{2+} in the protein extract. A, Mg^{2+} added to immunoprecipitation reaction increased subsequent light-regulated in vitro phosphorylation of phytochrome. Etiolated coleoptiles were irradiated with red light (R) for 1 s or 1 min, or exposed only to a green safelight (Gr). Mg^{2+} (10 mM) was added to protein extracts (containing 2 mM EDTA) prior to immunoprecipitation with anti-phytochrome where indicated above the gel lanes. Immunoprecipitates were washed and phosphorylated in vitro (with 10 mM Mg^{2+} added to the reaction as described in "Materials and Methods"), then proteins were electrophoresed and autoradiographed. The amount of phytochrome detected by Coomassie blue was similar for the different treatments (not shown). Arrowhead shows position of phytochrome. ^{32}P activity (cpm) of the excised phytochrome band is shown below each gel lane. B, A factor that stimulates phytochrome phosphorylation is present in the precipitate and depleted from the supernatant when Mg^{2+} is added to anti-phytochrome-precipitated proteins, but remains mostly in the supernatant in the absence of Mg^{2+}. Experiment as in A. Etiolated coleoptile protein extracts had 10 mM Mg^{2+} added (lane 1) or Mg^{2+} chelated with EDTA (lanes 2–4) when immunoprecipitates were formed. Immune complexes were reincubated with the same supernatant (lanes 1 and 4), the same supernatant to which Mg^{2+} was then added (lane 2), or a supernatant that had already been immunoprecipitated in the presence of Mg^{2+} (lane 3). The amount of immunoprecipitated phytochrome visible on Coomassie blue-stained gels was similar for the various treatments, but is not shown.

It is not clear whether the immunoprecipitated protein kinase described here is identical to the polycation-stimulated protein kinase co-purified with phytochrome by others, which also preferentially phosphorylates Pr rather than Pfr (Grimm et al., 1989; Kim et al., 1989; Wong et al., 1989). The co-purified protein kinase phosphorylates a Ser residue within the Ser-rich N-terminal tryptic dodecapeptide of oat phytochome, a different site than that phosphorylated by mammalian second messenger-dependent protein kinases (McMichael and Lagarias, 1990). The degree of preference of one form of phytochrome is much greater in immunoprecipitates (Figs. 4 and 5) than the slight preference the co-purified protein kinase has for Pr over Pfr (Wong et al., 1986, 1989). However, a variety of factors could modulate activity of immunoprecipitated protein kinase. For example, a co-immunoprecipitated protein could modify the phytochrome-phosphorylating activity, antibody binding might affect phytochrome conformation, or rapid extraction of immunoprecipitates might prevent a modification of protein kinase activity that occurred during phytochrome purification. Polycation stimulation of activity is characteristic of PAPK (Wong et al., 1989), but may be negligible in some preparations (Kim et al., 1989) and was not observed here. However, the highly basic IgG proteins present in all protein phosphorylation assays may have acted as a polycationic stimulus. The polyanionic protein kinase substrate casein (0.2 $\mu g/\mu L$), but not polycationic substrates such as histones, strongly inhibited phytochrome phosphorylation when they were added to immunoprecipitates (B.J. Biermann and L.J. Feldman, unpublished results), indicating that the immunoprecipitated protein kinase is charge sensitive like that co-purified with phytochrome. Phytochrome immunoprecipitates did not possess appreciable histone kinase activity, although this was reported in purified phytochrome preparations (Kim et al., 1989; Wong et al., 1989). It has been suggested that lack of specificity of kinase activity toward phytochrome in purified preparations is due to loss of kinase regulatory function during extraction (Kim et al., 1989). Immunoprecipitated protein kinase has substrate specificity not found in purified phytochrome preparations, and thus may be useful in the study of regulation.

Phytochrome phosphorylation was stimulated 5- to 10-fold when proteins were immunoprecipitated in the presence of Mg^{2+} (Fig. 7). This was due to enhanced co-immunoprecipitation of a stimulatory factor rather than to differences in the phytochrome itself, since immunoprecipitation supernatants contained less of the stimulatory factor when they were previously immunoprecipitated with Mg^{2+} but not when they were previously immunoprecipitated without Mg^{2+}. The simplest interpretation is that association of the protein kinase with phytochrome is enhanced by Mg^{2+}, although the possibility cannot be excluded that Mg^{2+} affects association of a factor that regulates the protein kinase. In any case, the large stimulation of phytochrome phosphorylation by co-immunoprecipitation of the protein kinase in the presence of Mg^{2+} suggests that phytochrome is not autophosphorylated. Phytochrome (from etiolated or irradiated coleoptile tissues) was not phosphorylated in several experiments where denatured peptides in anti-phytochrome immunoprecipitates (Pr or Pfr) were electrophoresed, renatured, irradiated with saturating red or far-red light, and then phosphorylated in the gel by the method of Klimczak and Hind (1990) (B.J. Biermann and L.J. Feldman, unpublished results). However, other protein kinases were autophosphorylated in these experiments. One interpretation is that another peptide, separated by electrophoresis, is required for phytochrome phosphorylation, but irreversible denaturation of an intrapeptide autophosphorylating activity cannot be ruled out as an explanation. Activity of the PAPK, however, was not irreversibly inhibited by exposure to detergents (Fig. 2).

The physiological role of the PAPK cannot be determined from the experiments described here, although some possibilities are suggested by parallels with known signaling systems involving receptor-mediated reversible protein phosphorylation. Like the regulatory regions or subunits of second messenger-dependent protein kinases, phytochrome could transduce signals through protein conformational changes that affect protein kinase activity of an associated catalytic region. Rather than binding of a second messenger, either photoactivation or ubiquitin-mediated proteolysis of phyto-

chrome would be the signal regulating protein kinase activity. Reversible phosphorylation of specific cellular substrates would then modulate cellular activities in response to light. However, there is no evidence that associated protein kinase activity differs between Pr and Pfr, as this model predicts. Saturating red or far-red irradiation did not affect in vitro phosphorylation of any endogenous proteins or added histones by protein kinases present in gel-filtered extracts of etiolated or light-grown (24 h of white light) coleoptiles. (Phosphoproteins were electrophoretically separated in one dimension, then autoradiographed [B.J. Biermann and L.J. Feldman, unpublished results].)

PAPK receptor- and conformation-specific activity resembles that of a group of kinases that phosphorylate G-protein-coupled receptors, such as the mammalian β-adrenergic receptor and light receptor, rhodopsin. Kinases that phosphorylate G-protein-coupled receptors do not directly transduce signals, but instead down-regulate activity of the stimulated receptor. Dephosphorylated light-activated rhodopsin is bound tightly by rhodopsin kinase (Buczylko et al., 1991). Light-activated rhodopsin is the only known substrate of rhodopsin kinase (Buczylko et al., 1991). Recently, evidence has been presented that G-proteins act downstream of phytochrome in transduction pathways leading to light regulation of gene transcription (Romero et al., 1991; Neuhaus et al., 1993; Romero and Lam, 1993).

Evidence that the protein kinase is intrinsic to phytochrome or is associated with high-affinity interactions (which would operate in vivo), together with the observed red/far-red light regulation and specificity of phytochrome phosphorylation, provide strong support for the hypothesis that PAPK modulates light responses in vivo. However, further studies are necessary to determine the physiological substrates and regulation of the protein kinase. The ability to use Mg^{2+} to associate or dissociate a phosphorylation-stimulating factor from phytochrome provides a tool for future biochemical characterization of this factor. Because immunoprecipitation allows very rapid extraction of PAPK, its activity may more closely resemble that in vivo than when it is purified with phytochrome. Assay of PAPK in multiple-tissue samples, for example tissues derived from signal-transduction mutants, would be greatly facilitated by immunoprecipitation. Biochemical information obtained from immunoprecipitation studies may complement genetic information derived from analysis of mutants deficient in phytochrome signal transduction (Chory et al., 1989; Deng et al., 1991) to define events leading from phytochrome photostimulation to regulation of gene transcription.

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