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A blue-light-activated GTP-binding protein in the plasma membranes of etiolated peas

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ABSTRACT **Heterotrimeric GTP-binding regulatory proteins (G proteins) have been identified as part of signal transduction systems in a wide variety of organisms. In this paper, we establish the presence of a G protein associated with the plasma membranes of the apical bud of etiolated peas. The GTPase activity is induced by low fluences of blue light administered to plasma membrane-enriched fractions. The activity is not responsive to red-light irradiation and is specific for GTP. The threshold for the excitation of the GTPase activity *in vitro* is $<10^{-1}$ μmol·m^{-2} of blue light, consistent with participation in the blue low-fluence system identified in the same tissue. A 40-kDa polypeptide is recognized by polyclonal antisera directed against the α subunit of the G protein transducin. The polypeptide also serves as a substrate for ADP-ribosylation by cholera and pertussis toxins. The ability of the 40-kDa polypeptide to serve as substrate for the toxin-mediated ribosylation is mediated by blue-light irradiation, implying that the 40-kDa polypeptide is the α subunit of a blue-light-stimulated G protein. The 40-kDa polypeptide binds a nonhydrolyzable photoaffinity-labeling analog of GTP only after irradiation with blue light. The protein we have described may function as an α subunit of a G protein active in the process of light-mediated development in higher plants.**

The signal transduction mechanisms governing environmentally stimulated events in plants are not well understood. Blue-light-regulated events are evolutionally ancient and ubiquitous, and blue light is required by higher plants for growth and development (1). *Pisum sativum L.*, the common garden pea, has well-characterized responses to blue light, regulated by either or both of two blue-light-responding photoreception systems (2–7). The blue low-fluence system has a threshold to low fluences of blue light (at or below 10^{-1} μmol·m^{-2}), whereas the blue high-fluence system has a threshold to high fluences of blue light (between 10^2 and 10^3 μmol·m^{-2}) (2–7). Excitation of either blue-light system will affect the rate of transcription for specific nuclear gene families immediately upon irradiation and in the absence of protein synthesis (e.g., excitation of the blue low-fluence system induces *Cab* gene transcription) (2, 3) as well as basic growth events (e.g., excitation of the blue low-fluence system causes suppression of epicotyl elongation) (4, 5). Dark-grown seedlings exhibit only activity of the blue low-fluence system (4, 5), making it convenient to study the signal transduction mechanism for this blue-light system. The nature of the receptor and signal transduction mechanism for any of these blue-light effects remains unknown.

Heterotrimeric GTP-binding regulatory proteins (G proteins) act as intermediaries in signal transduction pathways consisting of a plasma membrane receptor, a G protein, and an effector system (8). Heterotrimeric G proteins have α (39–46 kDa), β (35–37 kDa), and γ (8 kDa) subunits (8). In general, the α subunit possesses the unique activities ascribed to G proteins, including (*i*) the ability to bind GTP, (*ii*) an intrinsic GTPase activity, (*iii*) recognition by bacterial toxins as a substrate for ADP-ribosylation, (*iv*) recognition of a specific plasma membrane receptor, and (*v*) recognition of a specific effecter system (8). It has been demonstrated that blue-light irradiation of a plasma membrane-enriched fraction derived from pea epicotyls will result in altered phosphorylation for several membrane polypeptides (9, 10). These data suggest that a blue-light receptor resides in the plasma membrane. It is possible that the receptor responsible for the blue-light responses we have previously described for the developing leaf tissue (3, 6, 7) is also located in the plasma membrane. As many of the characterized plasma membrane-bound receptors in animal systems are associated with signal transduction paths containing a G protein it is possible that the blue-light receptor may also couple to a G protein. We have identified a blue-light-activated heterotrimeric GTP-binding regulatory protein associated with plasma membranes isolated from the apical buds of dark-grown peas.

MATERIALS AND METHODS

Plasma Membrane Isolation and Irradiation. Seedlings of *P. sativum* L. cv. Alaska were grown in absolute darkness (3) for 7 days after which the apical buds were harvested and the plasma membranes were purified (10) under a green safelight (3). The membranes are stored in the dark until used in the various assays. The blue-light and red-light sources are described elsewhere (3, 5). Unirradiated plants received a mock irradiation—that is, they were treated exactly as the irradiated plants except that the shutter on the light source was never opened.

For all figures data are derived from three to five independent membrane isolations. Each independent membrane isolation (derived from ≈1000 seedlings) was made from seedlings that were grown during nonoverlapping periods of time. The variance was between 5% and 15%.

GTPase Activity. Reactions, unless indicated, were carried out at 4°C in the presence of 1.0 nM [γ-^{32}P]GTP, 1.0 μM GTP, and 10 μg of plasma membrane protein per ml. The GTP mixture was added to the membranes either coincident with the onset of irradiation (Fig. 1*A*) or 30 s prior to irradiation (Fig. 1 *B* and *C*). All other conditions are described elsewhere (11). Aliquots were removed at the times indicated and the GTPase activity was determined by measuring the amount of labeled orthophosphate produced. Orthophosphate was precipitated by incubation at 4°C for 15 min in 0.1 M perchloric acid/15 mM ammonium molybdate/5 mM tetraethylammonium hydrochloride, pH 5.0. The precipitate was collected on GFC filters (Whatman), washed with the precipitating buffer,

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Abbreviations: GTP[γ-^{35}S], guanosine 5′-[γ-^{35}S]thiotriphosphate; ROS, rod outer segment.

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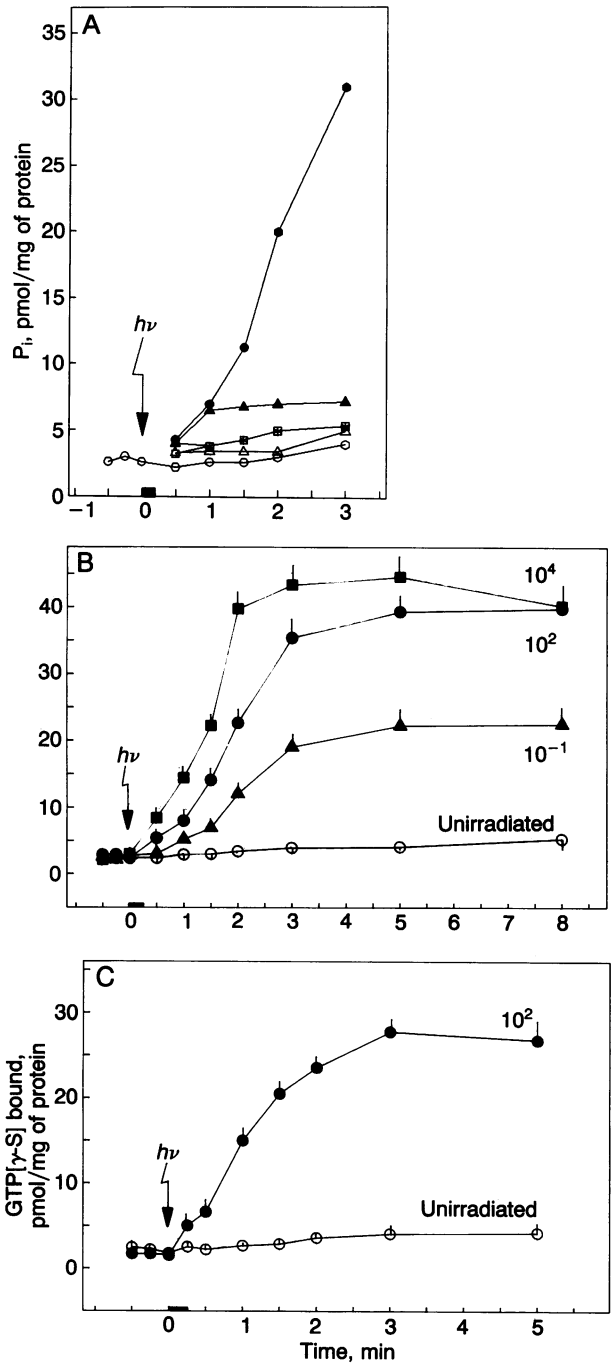

FIG. 1. Low-fluence blue light induces a GTPase activity in the plasma membranes from the apical buds of 7-day-old dark-grown peas. The plasma membranes from the apical buds of 7-day-old dark-grown peas were isolated and maintained under safe light. The purified membranes were assayed for GTPase activity under different light and substrate conditions. The onset of the 15 s of irradiation is indicated by the arrow; the box on the time axis indicates the duration of the irradiation. (*A*) The GTPase activity of purified membranes was measured after irradiation with blue light (●; total fluence of 10^2 μmol·m^{-2} delivered in 15 s), red light (▲; total fluence of 10^2 μmol·m^{-2}, delivered in 15 s), or no light (○). GTPase assays with blue-light-irradiated membranes were also performed in the presence of a 25-fold excess (25 μM) of the GTP-specific analog GTP[γ-S] (△) or guanylyl imidodiphosphate (GppNHp; □). In a separate assay using blue-light-irradiated membranes the 1.0 μM GTP was replaced by 1.0 μM ATP (*). (*B*) The fluence of blue light necessary to elicit the GTPase activity was determined by irradiating the dark-maintained membranes with a 15-s pulse of either 10^{-1} (▲), 10^2 (●), or 10^4 (■) μmol·m^{-2} of blue light or no blue light (○). (*C*) The ability of the membrane proteins to bind GTP[γ-S] was tested as a function of blue-light irradiation (●). Control plants received no blue light (○). Aliquots were removed at the times indicated and the amount of GTP[γ-S] bound was determined. Blue-light irradiation was 15 s long and contained a total fluence of 10^2 μmol·m^{-2}.

and assayed for radioactivity by liquid scintillation spectroscopy.

Antibody Studies. Plasma membrane proteins were separated on 12.5% polyacrylamide gels, electroblotted onto nitrocellulose membranes, and used for immunodetection. The preparation and characterization of Tα and Tβγ purified from bovine retina and anti-Tα and anti-Tβγ antisera are described elsewhere (12, 14). The preparation and characterization of the GO/1 antiserum (obtained from Alan Spiegel, National Institutes of Health), which is directed against a synthetic decapeptide of $G_o\alpha$ and $G_{i3}\alpha$, is also described elsewhere (13). Bovine rod outer segment (ROS) membranes were isolated as described elsewhere (14).

Guanosine 5′-[γ-S]Thiotriphosphate (GTP[γ-S]) Binding. GTP[γ-S] binding assays were performed as described by Hamm and Bownds (16). Reaction buffer containing 1.0 μM GTP[γ-S] and 1.0 nM GTP[γ-^{35}S] was added to the membranes (10 μg/ml) 30 s before the onset of blue-light irradiation. Aliquots were removed at the times indicated, precipitated onto nitrocellulose filters (type HA, 45 μm, Millipore), washed, and assayed for radioactivity by liquid scintillation spectroscopy. The use of glass microfiber filters, which retain only membrane fragments, lessened the detected amount of GTP[γ-^{35}S] bound for irradiated and unirradiated plasma membranes by ≈20%.

ADP-Ribosylation by Pertussis and Cholera Toxins. Assay conditions for both toxins were identical and are described elsewhere (15, 17). Loosely associated peripheral proteins (referred to as Sol in Fig. 3) were obtained by forcing plasma membranes through a Pasteur pipette tip, washing with toxin assay buffer, and recentrifugation. The pellet was used as the membranes and the supernatant was used as the source for a possible ADP-ribosylation factor (15). Incubation of the membranes with the toxin was terminated after 75 min by precipitation in trichloroacetic acid. Proteins were denatured and separated on 12.5% polyacrylamide gels. ADP-ribosylated proteins were detected by autoradiography.

Photoaffinity Labeling. Photoaffinity labeling was performed as described by Gordon and Rasenick (18). Fifty micrograms of plasma membrane protein was incubated with 0.1 μM P^3-(4-azidoanilido)-P^1 5′-[α-^{32}P]GTP (the photoaffinity label) for 5 min in the presence or absence of blue-light irradiation (10^1 μmol·m^{-2}·s^{-1}) followed by 3 min of UV light.

RESULTS

Blue-Light Activation of a GTPase Activity. The plasma membranes of the apical buds of 7-day-old dark-grown peas were tested for their ability to specifically hydrolyze GTP as a result of blue-light irradiation. The data in Fig. 1*A* demonstrate that treatment of purified pea plasma membranes with a brief (15 s) pulse of low-fluence blue light (total fluence of 10^2 μmol·m^{-2}) will activate a GTPase activity. The GTPase activity is not elicited by an equal fluence of red light, indicating that the GTPase activity is not controlled by the red-light photoreceptor, phytochrome.

Two assays were performed to determine if the GTPase activity is specific to GTP. The GTP was replaced with either ATP or one of the nonhydrolyzable GTP analogs, guanosine thiotriphosphate (GTP[γ-S]) and guanylyl imidodiphosphate (GppNHp), both competitive inhibitors of the GTPase activity (8). The data shown in Fig. 1*A* indicate that the GTP analogs, when used in a 25-fold excess, compete with GTP (final concentration, 1.0 μM) and eliminate the blue-light-

stimulated GTPase activity. Blue light fails to stimulate an ATPase activity, providing further confirmation of the specificity for GTP.

The apparent K_m values for the GTPase activity in the blue-light-treated and control plasma membrane fractions are 1.37 ± 0.24 μM and 37.8 ± 10.2 μM, respectively (not shown).

To determine the threshold and saturation fluences for this blue-light-dependent GTPase activity we measured the GTPase activity at three different fluences. The data shown in Fig. 1*B* demonstrate that the threshold for the blue-light stimulation of the GTPase is $<10^{-1}$ μmol·m^{-2} of blue light. This is in keeping with the threshold observed for the blue low-fluence system (2, 4–7) and consistent with the presence of the blue low-fluence system in the apical buds of etiolated peas (4, 6).

The data also indicate that the blue-light-stimulated GTPase activity terminates 3 min after the light treatment regardless of the fluence tested. This result indicates that the GTPase activity is terminated by a time-dependent mechanism, contrasting the fluence-dependent activation of the GTPase activity.

To confirm that a protein or proteins in the plasma membrane can bind GTP as a result of blue-light irradiation at low substrate concentrations, we measured the ability of the plasma membrane fraction to bind GTP[γ-^{35}S] in a blue-light-dependent manner. Fig. 1*C* illustrates that blue light enhances the binding of GTP[γ-S] to the plasma membrane fraction ≈5-fold over that of the unirradiated plasma membrane fraction. It is noteworthy that although GTP[γ-S] binds to the plasma membrane fraction in a blue-light-dependent manner at low concentrations of GTP[γ-S] (1.0 μM), at substrate concentrations exceeding the K_m (100 μM), irradiated and unirradiated plasma membrane fractions bind GTP[γ-S] equally well (data not shown).

Antibody Cross-Reactivity. The above data establish that one or several proteins, probably in the plasma membrane, are capable of binding and hydrolyzing GTP and will only do so after the membranes have been irradiated with blue light. These data strongly suggest that one or several blue-light-activated G proteins exist in the plasma membrane. To determine if the plasma membranes of dark-grown peas contain α subunits of heterotrimeric G proteins we tested for cross-reactivity with antisera developed against the α subunits of recognized G proteins.

The data shown in Fig. 2 indicate that polyclonal antisera raised against characterized G proteins recognize specific polypeptides in the plasma membrane of the dark-grown pea buds. Anti-Tα recognizes 40-kDa and a 30-kDa polypeptides (lane 2); presorption with pure Tα eliminates detection of both polypeptides (lane 3). The polyclonal antiserum GO/1, which detects $G_o\alpha/G_{i3}\alpha$ (13), detects a single 40-kDa polypeptide in pea plasma membranes (lane 4). These data suggest that one or several 40-kDa polypeptides in the pea plasma membrane represent a polypeptide with structure similar to the α subunits of other established heterotrimeric G proteins.

Polyclonal antibodies raised against Tβγ also recognize several polypeptides in the plasma membranes of dark-grown peas. The polypeptides recognized have apparent molecular masses of 46, 36, 32, and 26 kDa (lane 5); detection was nearly eliminated by presorption with pure Tβγ (lane 6). The specificity of the anti-Tα and anti-Tβγ antisera for Tα and Tβγ and the efficiency of the presorption protocol are demonstrated using the proteins of the bovine ROSs (lanes 7–12). The polypeptides recognized by the respective antibodies correspond to the correct molecular masses for the respective antigens, and presorption with purified antigen eliminates the antibody staining.

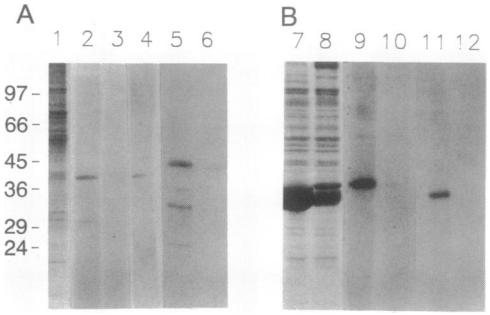

FIG. 2. Recognition of specific polypeptides within the plasma membranes of the apical buds of 7-day-old dark-grown peas by antibodies directed against the α subunit of various GTP-binding regulatory proteins, including transducin and the βγ subunits of transducin. (*A*) The plasma membrane proteins from the apical buds of 7-day-old dark-grown peas were tested for cross-reactivity against antibodies directed against the various subunits of transducin and a decapeptide derived from the α subunit of G_o. Lanes shown represent Coomassie stain (lane 1) and immunoblots incubated with the following: anti-Tα antiserum (lane 2), anti-Tα antiserum following presorption with purified Tα subunit (lane 3), GO/1 antiserum (lane 4), anti-Tβγ antiserum (lane 5), anti-Tβγ antiserum following presorption with purified Tβγ subunits (lane 6). The data shown represent one of several replicate experiments, each using membranes derived from plants grown during separate, nonoverlapping time periods. Molecular masses are given in kDa. (*B*) Bovine ROS membrane proteins were electrophoresed and used in immunodetection assays as in *A*. Lanes shown represent the Coomassie stain of nonheated (lane 7) and heated (2 min at 90°C, lane 8) ROS polypeptides; lanes 9, 10, 11, and 12 correspond exactly to the immunodetection treatments described for lanes 2, 3, 5, and 6, respectively.

ADP-Ribosylation by Pertussis and Cholera Toxins. To support the notion that the 40-kDa polypeptide(s) identified by the GO/1 and anti-Tα antibodies represents an α subunit of a heterotrimeric G protein we sought to determine if the polypeptide would be recognized by pertussis and/or cholera toxins. Characterized α subunits of G proteins are often substrates for covalent modification by either or both of the secretory toxins of *Bordetella pertussis* and *Vibrio cholerae* (8). Pertussis and cholera toxins transfer an ADP-ribose moiety to a specific cysteine and arginine, respectively.

Furthermore, the two toxins have contrasting preferences with respect to the conformation of the α subunit such that ADP-ribosylation by pertussis toxin occurs more efficiently when the G protein is in the nonexcited (GDP bound) state, whereas ADP-ribosylation by cholera toxin occurs more efficiently when the G protein is in the excited (GTP bound) state. Thus, through use of the two toxins we could not only test by two additional methods whether the 40-kDa polypeptide is an α subunit but we could also determine if its conformation is altered by irradiation with blue light.

The data shown in Fig. 3*A* indicate that pertussis toxin (lanes 2–9) recognizes a single 40-kDa polypeptide, the same molecular mass as identified by the antibodies. Furthermore, labeling by the pertussis toxin will occur unless light and GTP are present (lanes 3 and 6), suggesting that light and GTP are necessary for activation of this putative pea G protein. Addition of the peripheral membrane protein fraction (see *Materials and Methods* for definition; referred to as Sol in Fig. 3) enhances the amount of pertussis labeling (for example, compare lanes 2 and 5), suggesting the presence of an ADP-ribosylating factor similar to those described for other systems (16). The peripheral protein fraction itself has no substrate for the pertussis toxin (lane 4). The 40-kDa polypeptide is not labeled in the absence of pertussis toxin and the lower molecular mass band present in lane 6 is due to autoribosylation of the pertussis toxin (not shown).

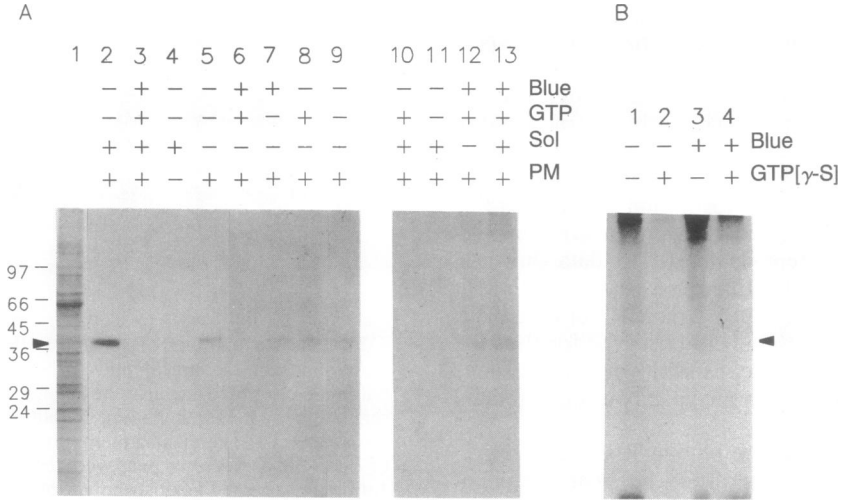

FIG. 3. Recognition of specific membrane polypeptides by cholera and pertussis toxins and the UV cross-linking, nonhydrolyzable GTP analog P^3-(4-azidoanilido)-P^1 5′-GTP in response to blue-light irradiation. (*A*) The ability of pertussis and cholera toxins to ribosylate plasma membrane (PM) proteins from the apical buds of 7-day-old dark-grown peas was tested as a function of blue-light irradiation and peripheral membrane proteins. Lane 1 represents the Coomassie-stained gel. Pertussis toxin (lanes 2–9) or cholera toxin (lanes 10–13) was incubated with 45 μg of plasma membrane protein and 5 μg of peripheral membrane proteins (lanes 2 and 3, pertussis; lanes 10, 11, and 13, cholera; labeled Sol), 50 μg of plasma membrane protein (lanes 5–9, pertussis; lane 12, cholera), or 5 μg of peripheral membrane protein alone (lane 4, pertussis). Pertussis toxin assays were done in the dark with no GTP except lanes 3 and 6, which were done in continuous blue light (10^{-1} μmol·m^{-2}·s^{-1}) with 100 μM GTP, and lanes 7 and 8, which were done in continuous blue light or with GTP, respectively. Cholera toxin assays were done in continuous blue light (10^{-1} μmol·m^{-2}·s^{-1}) with 100 μM GTP (lanes 12 and 13) or in complete darkness with (lane 10) or without (lane 11) GTP. The arrowhead indicates the 40-kDa polypeptide. Molecular masses are given in kDa. (*B*) Plasma membrane proteins from the apical buds of 7-day-old dark-grown peas were tested for the ability to bind the radiolabeled, nonhydrolyzable, photoaffinity GTP-specific analog P^3-(4-azidoanilido)-P^1 5′-[α-^{32}P]GTP as a function of blue-light irradiation. Plasma membrane proteins were incubated with 0.1 μM P^3-(4-azidoanilido)-P^1 5′-[α-^{32}P]GTP for 5 min either without (lanes 1 and 2) or with blue light (lanes 3 and 4), followed by 3 min of UV light. Proteins were precipitated, solubilized, separated, and detected as in *A*. In lanes 2 and 4, 10.0 μM GTP[γ-S] was included to compete with the binding of P^3-(4-azidoanilido)-P^1 5′-[α-^{32}P]GTP.

Curiously there are no peptides recognized by the pertussis toxin of a size consistent with an α subunit when light and GTP are present (lanes 3 and 6). However, there do exist many characterized α subunits that are not recognized by pertussis toxin (19). Furthermore, it is possible that one or several α subunits may have been lost during the membrane isolation. This is known to occur for several characterized α subunits (e.g., ref. 20).

The data for ribosylation by cholera toxin (lanes 10–13) confirm those of the pertussis toxin. Cholera toxin will ADP-ribosylate a 40-kDa polypeptide only when blue light and GTP are supplied (lanes 10 and 11 compared with lanes 12 and 13).

Photoaffinity Labeling. The GTPase assay suggests that a blue-light-activated GTP-specific GTPase activity exists within the plasma membranes derived from the apices of dark-grown peas. Binding studies with the nonhydrolyzable GTP analog GTP[γ-S] indicate that one or several proteins within the membrane are capable of blue-light-induced binding of GTP. The anti-Tα and GO/1 antibody cross-reactivity and the ribosylation by pertussis and cholera toxins all concur that a 40-kDa polypeptide in the plasma membranes of dark-grown pea apices behaves as the α subunit of a heterotrimeric G protein. Furthermore, the blue-light effects on the ability of the pertussis and cholera toxin activities suggest that the 40-kDa polypeptide is the α subunit of a blue-light-activated G protein. If the 40-kDa polypeptide is indeed the α subunit of a blue-light-activated heterotrimeric GTP-binding protein, then it should bind GTP in a blue-light-dependent manner.

To determine if the 40-kDa polypeptide shows blue-light-induced binding of GTP, we have used the UV cross-linking, nonhydrolyzable GTP analog P^3-(4-azidoanilido)-P^1 5′-GTP (18, 20). The data shown in Fig. 3*B* demonstrate that blue light induces the binding of the photoaffinity label to a 40-kDa polypeptide (lane 3 vs. lane 1), indicating that a 40-kDa polypeptide in the plasma membrane will bind GTP as a function of blue-light irradiation. To ensure that the binding of the photoaffinity label was specific for GTP we also assayed in the presence of excess amounts of the nonhydrolyzable GTP analog GTP[γ-S]. Inclusion of the analog eliminated binding of the photoaffinity label by the 40-kDa polypeptide, indicating that the 40-kDa polypeptide specifically binds GTP (Fig. 3*B*, lanes 1 and 3 vs. lanes 2 and 4, respectively). Thus, the 40-kDa polypeptide can bind GTP and only as a result of blue-light irradiation.

DISCUSSION

The data presented herein suggest strongly that there is a blue-light-activated, heterotrimeric, GTP-binding regulatory protein in the plasma membrane of apical buds of dark-grown peas. The data also suggest that the α subunit of this G protein is a 40-kDa polypeptide. Blue light is required for complete development of the pea plant and it is possible that this G protein plays a role in the signal transduction system(s) through which these developmental responses are elicited.

The activity of this G protein has a threshold to blue light in the low-fluence range and is not elicited by red light. Because the threshold is in the low-fluence range and because dark-grown peas only exhibit the blue low-fluence system activity (4, 7), it is likely that this G protein functions as part of the signal transduction mechanism defining the blue low-fluence system. This photomorphogenic system is known to regulate many events during plant development, including the rate of transcription for several nuclear coded genes (2, 3). In the case of the *Cab* gene family, the regulation occurs immediately upon blue-light regulation and in the absence of protein synthesis (2).

To our knowledge, a biological role for a G protein in a higher plant has not been reported previously. A cDNA clone for a putative α subunit has been obtained from *Arabidopsis*

thaliana (21); however, no biological role has been described for the putative α subunit. The predicted molecular mass and tissue localization data suggest that the *Arabidopsis* α subunit is not that which we describe for the apical buds of dark-grown pea. Several other groups have reported the presence of GTP/ATPase activities in the plasma membranes of various plant species and/or the presence of polypeptides capable of ribosylation by pertussis toxin (22–25). Again, no biological role has been described for these putative G proteins.

The blue-light-regulated G protein we describe has several properties in common with transducin, the G protein that functions in the retina; both are light activated and, unlike many other G proteins, both are ribosylated by pertussis and cholera toxins. The full extent of the similarity and the specific properties of the blue-light-activated G protein will await purification and characterization of the pea G protein.

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