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Protein phosphorylation in isolated nuclei from etiolated *Avena* seedlings

Effects of red/far-red light and cholera toxin*

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We have studied the phosphorylation/dephosphorylation of several nuclear proteins in isolated nuclei from etiolated *Avena* seedlings as a function of red/far-red light. The effect of stimulatory (ADP-ribosylation by cholera toxin) or inhibitory (GDPβS) conditions for GTP-binding proteins was also studied. Red or far-red light enhanced the phosphorylation level of 2 nuclear proteins with molecular masses of 75 and 60 kDa. The phosphorylation pattern was affected by the addition of cholera toxin or GDPβS to the isolated nuclei. At least 2 proteins with molecular masses of 24 and 75 kDa cross-reacted by Western blot with GTP-binding protein antibodies.

Signal transduction; G-protein; Phytochrome; Nuclei; *Avena sativa*

1. INTRODUCTION

Protein phosphorylation plays an important role in regulatory and signal transducing processes in plants [1]. In the photomorphogenesis of higher plants, phytochrome might trigger a light signal transduction cascade by modulating protein phosphorylation/dephosphorylation [2]. For example, phosphorylation of nuclear proteins is implicated in the regulation of phytochrome-mediated light-responsive gene expression. The AT-1 transcription factor that binds to the promoters of some phytochrome-mediated light-responsive genes in pea is reversibly phosphorylated [3]. In an accompanying paper, we examined the possible role of G-proteins in the phytochrome-mediated signal transduction.

In mammalian cells, G-proteins and their functional roles in signal transduction are well known [4]. There are also small molecular mass proteins (20–25 kDa) such as *ras* oncogene-encoded proteins and other *ras*-related gene products [5]. These proteins transduce environmental and metabolic signals from a wide variety of receptors to specific biochemical effectors. For example, transducin couples light activation of rhodopsin with cGMP phosphodiesterase in vertebrate retina [4]. G-Proteins undergo a covalent modification which alters their regulatory functions. For example, ADP-ribosylation catalyzed by cholera toxin inhibits the GTPase activity of GTP-binding proteins, thereby trapping the proteins in their active conformational state [4,6]. In contrast to GTP-binding proteins in animal cells, very little is known about G-proteins in plants. Blum et al. [7] detected G-proteins in the plasma membrane of *Vicia faba*, *Arabidopsis thaliana*, and *Commelina communis*. The presence of GTP-binding proteins has also been reported in *Lemna paucicostata* [8], in spinach thylakoid membranes [9] and in a zucchini hypocotyl [10]. Recently, 2 genes with homology to mammalian GTP-binding proteins, have been cloned in *Arabidopsis thaliana*, namely the GPA1 gene coding for an α-subunit-like G-protein [11] and the *ara* gene homologous to the *ras*-related gene family [12].

We present herein a preliminary study involving the phosphorylation/dephosphorylation of nuclear proteins regulated by light and evidence that these processes are affected by modulators of mammalian G-proteins. The presence of a high molecular weight G-protein-like substance has also been described.

2. MATERIALS AND METHODS

2.1. *Plant material*

Avena sativa L. seedlings (cv Garry oat; Agriculver Co., Trumansburg, NY) were grown in the dark on moist vermiculite for

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Abbreviations: Cab, chlorophyll a/b binding protein gene; GTP-binding proteins or G-proteins, guanine nucleotide binding proteins; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); *phy*, phytochrome gene.

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3.5 days at 25°C and harvested under dim green light, as described previously [13].

2.2. *Nuclei isolation*

Intact oat nuclei were isolated from etiolated oat seedlings, according to the method of [14]. The isolation was performed under very dim green light (fluence rate of 9.8×10^{-8} W/cm^2 provided by a 20 W cool white fluorescent lamp covered with 2 layers of green plexiglass filter No. 874 from Dallas Stage Lighting Equipment, Dallas, TX). Nuclei were assayed by 4,6-diamino-2-phenylindole (DAPI; 0.1 μg/ml) staining and examined under a fluorescent microscope.

2.3. *SDS polyacrylamide gel electrophoresis and Western blot analysis*

Slab gel electrophoresis was performed by the method described by [15] using a 10%–20% linear gradient running gel and a 4% stacking gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes. Transfer buffer containing 192 mM glycine, 25 mM Tris base and 20% methanol was used. Complete transfer was achieved at 100 mV for 12–16 h at 4°C. After transferring the proteins, the lane containing molecular weight markers was removed and stained with 0.5% Ponceau red (dissolved in 1% acetic acid). The rest of the membrane was immersed for 2 h in incubation solution containing 5% bovine serum albumin in phosphate-buffered saline (PBS; 0.9% NaCl in 10 mM sodium phosphate, pH 7.2). Primary antibodies specific for Gi, Gs, transducin (SA/1) and for the GTP-binding domain (GA/1) (NEN Research Products, USA) were used at 1:500 dilution in incubation buffer and incubated overnight. The membranes were washed 3 times with PBS containing 0.05% Nonidet-P40 (NP-40) and twice with PBS. For the enzyme coupling, a protein A-horseradish peroxidase conjugate was used at 1:1000 dilution, and incubated for 2 h. The membranes were then washed as described above. The blot was developed by placing the membranes in a substrate solution containing 25 mg diaminobenzidine, 340 mg imidazole and 50 μl of 30% H_2O_2 in 50 ml PBS.

2.4. *Phosphorylation of nuclear proteins*

The reaction mixture (50 μl total volume) containing 50 mM HEPES, pH 6.8, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 20% glycerol, 1 mM PMSF and 10^5 nuclei was incubated with 10 μCi [γ-^{32}P]ATP, 3000 Ci/mmol (NEN Research Product, USA) for 10 min at 25°C. The reaction was stopped by adding 25 μl of electrophoresis sample buffer [15] and boiling the samples for 5 min. Reactions were performed under dim green light. Nuclei were irradiated with red or far-red light where indicated. Diode lasers (Melles Griot) of 670 nm (red) or 750 nm (far-red) were used for irradiation. Samples were irradiated (as indicated) for 60 s before the start of the reaction. The proteins were separated by SDS-PAGE. The gel was then dried onto Whatman 3MM paper and subjected to autoradiography on Kodak X-Omat AR film with a DuPont intensifying screen at −70°C.

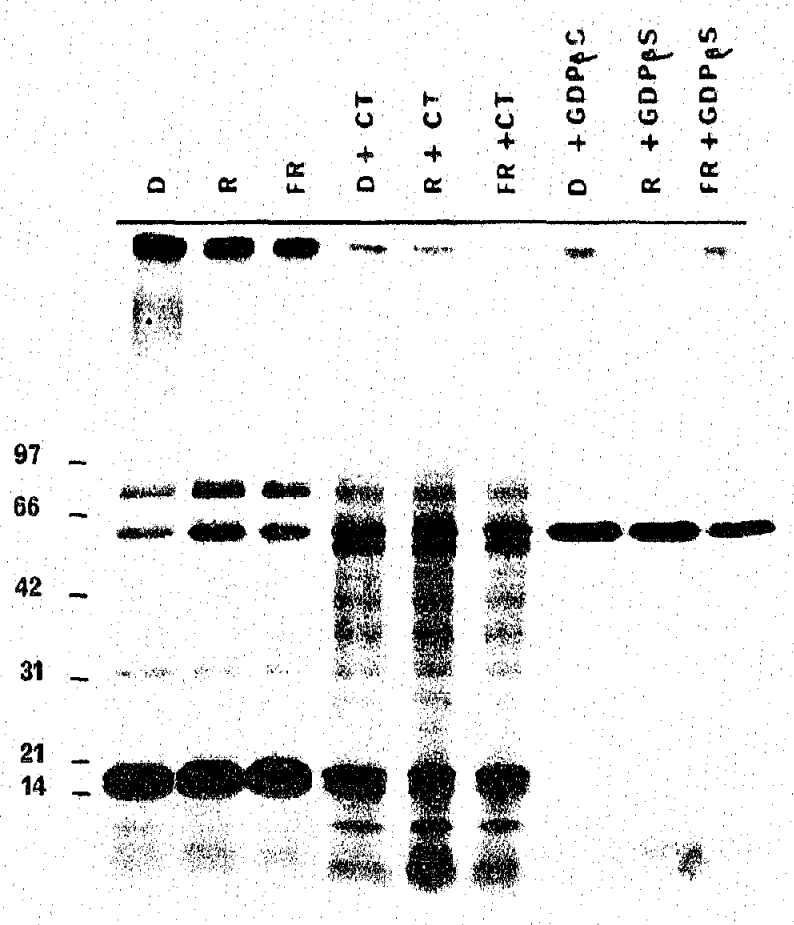

Fig. 1. Phosphorylation of nuclear protein in the presence of cholera toxin and GDPβS. Nuclei isolated from etiolated oat seedlings were incubated 10 min in the dark at 25°C with 10 μCi of [γ-^{32}P]ATP. The nuclei were treated using the following conditions before the incubations: D, darkness; R, irradiated 1 min with red light; FR, irradiated 1 min with far-red light; D + CT, D in the presence of cholera toxin (0.5 mg/ml) and NAD^+ (20 μM); R + CT, R in the presence of cholera toxin and NAD; FR + CT, FR in the presence of cholera toxin and NAD; D + GDPβS, D in the presence of GDPβS (0.5 mM); R + GDPβS, R in the presence of GDPβS; FR + GDPβS, FR in the presence of GDPβS. Molecular weight markers are indicated on the left; molecular masses 97.4 kDa, 66.2 kDa, 42.7 kDa, 31.0 kDa, 21.5 kDa and 14.4 kDa (from top to bottom).

3. RESULTS

Protein phosphorylation was studied as a function of light (phytochrome) and factors that affect the activity of G-proteins. Irradiation of the nuclei with red or far-red light pulses resulted in an increase in phosphorylation of 2 proteins with molecular masses of 60 and 75 kDa, as shown in Fig. 1. Red light appears to elicit an increased level of phosphorylation compared to far-red light. Red or far-red light treatment dephosphorylated both 10 and 68 kDa proteins (Fig. 1).

Cholera toxin catalyzes ADP-ribosylation of G-proteins. Cholera toxin treatment of *Avena* seedling nuclei in the dark markedly affected the phosphorylation pattern, compared to the dark control without cholera toxin treatment (compare 4th lane with 1st lane, Fig. 1). In particular, phosphorylation of the 75 kDa protein band appears to decrease, while phosphorylation of the 10 and 60 kDa bands was intensified. Additional proteins in the molecular mass range of 25–52 kDa were phosphorylated in the presence of cholera toxin. Red or far-red light treatment did not show a significant effect on the cholera toxin-catalyzed phosphorylation in the dark, except for an enhanced phosphorylation of a 10 kDa protein by red light. Cholera toxin markedly reduced the phosphorylation level for the 16 and 18 kDa protein bands (Fig. 1; compare first 3 lanes with middle 3 lanes).

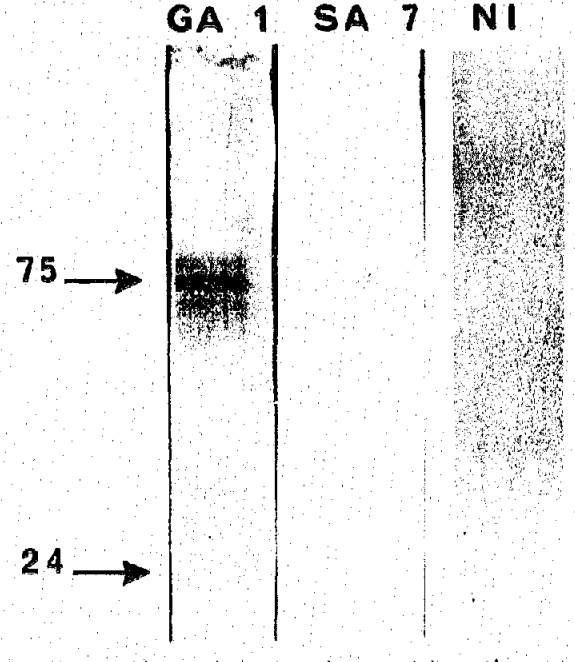

Fig. 2. Western blot analysis. Samples containing 10^4 nuclei were separated by SDS electrophoresis in a 10%–20% polyacrylamide gel and blotted onto a nitrocellulose membrane. The filters were incubated with GA/1, SA/7 antibodies or control serum (NI; non-immune) for 12–16 h. The blot were developed with a protein A-horseradish peroxidase conjugate and diaminobenzidine. The molecular weights of the proteins were calculated from molecular weight markers.

The addition of GDPβS, an inhibitor of GTP-binding proteins [16], resulted in the inhibition of the radioactive labeling of all the proteins except the 60 kDa protein, which was heavily phosphorylated under these conditions (Fig. 1). The increase in phosphorylation of the 60 kDa protein for dark control and red light treated nuclei (7th and 8th lanes, respectively) in the presence of GDPβS was measurably reduced by far-red light treatment (last lane).

To explore the putative relationship between protein phosphorylation (Fig. 1) and the involvement of GTP-binding proteins in nuclei, nuclear proteins were separated on an SDS electrophoresis gel followed by electrotransfer of the proteins to a nitrocellulose membrane. The blot was incubated with antibodies against the conserved GTP-binding domain moiety (GA/1) and other antibodies specific for Gi, Gs and transducin (SA/1). Antibodies have been used to analyze similarity between different species as well as to detect G proteins in other plants [7,17]. The Western blot analysis (Fig. 2) showed a prominent band with apparent molecular weight of 75 000. In addition, the GA/1 antibody also recognized a minor band at 24 000.

4. DISCUSSION

Phosphorylation of nuclear proteins has been reported in several plants and appears to be involved in several physiological responses [1–3,18]. The level of phosphorylation of nuclear proteins, particularly 60 and 75 kDa, in nuclei isolated from etiolated oat seedlings was apparently modulated by red and far-red light (Fig. 1). Red/far-red enhancement/modulation in the phosphorylation level of several nuclear proteins has also been observed in pea nuclei [18]. The fact that both red and far-red light pulses were effective in phosphorylating 60 and 75 kDa proteins is suggestive of phytochrome involvement as a very low fluence rate response (VLFR). The phosphorylation of the other nuclear proteins was independent of the light conditions.

The phosphorylation process was affected by modulators of mammalian G-proteins (cholera toxin and GDPβS). The light-dependent phosphorylation of the 60 and 75 kDa proteins was significantly blocked by cholera toxin. The inhibitor GDPβS, stimulated and inhibited the labeling of the 60 kDa and 75 kDa bands respectively, indicating that both proteins are phosphorylated by different kinases or regulatory mechanisms. Although there is no evidence for nuclear location of the phytochrome molecule, this result suggests that the phosphorylation of these 2 nuclear proteins may be regulated by a light-dependent G-protein cascade. The presence of G-proteins in plants has been reported in several plant species. Their possible involvement in the phytochrome-mediated signal transduction has been suggested by the effect of cholera toxin on the

light-regulated expression of the *phy* and *Cab* genes in intact plant (accompanying paper [19]).

The light-independent phosphorylation of the proteins with molecular masses of 10 and 58 kDa was stimulated by cholera toxin (and to a lesser extent, other proteins in the molecular mass range of 21–60 kDa were also phosphorylated). On the other hand, the incorporation of ^{32}P-radioactivity into the 16 and 18 kDa proteins was inhibited by the toxin. These proteins may also be regulated by a G-protein cascade, but their involvement in a signal transduction mechanism is speculative at the present time.

The presence of α-subunit-like polypeptides as well as small *ras*-like proteins has been reported by using antibodies against mammalian G-proteins [7,19]. The estimated molecular weight (75 000) of the GA/1-recognized G-protein shown in Fig. 2 is different from the typical α-subunit of known G proteins with a molecular weight of 40 000–50 000 [1,2]. There appear to be additional G-protein bands with molecular masses near the 75 kDa band (Fig. 2). It is possible that the concentration of a 75 kDa protein was too low to be detected in the crude extract, compared to its concentration in the nuclei preparation (see below).

We have no evidence that the 75 kDa G-protein-like protein is responsible for the effects described above. Although, in general, GTP-binding proteins are localized in the plasma membrane, GTP-binding proteins have also been found in mammalian nuclear envelopes with a wide range of molecular masses (20–140 kDa) [20]. The method used for the plant nuclei isolation yields intact nuclei [14]. In fact, the effect of phytochrome on RNA transcription in isolated nuclei has been observed with or without exogenously added phytochrome [14]. This observation could be interpreted to suggest that at least part of the signal transduction chain is localized in the nuclei. The effect we observed may be due to the presence of very low amounts of cytosolic phytochrome in the nuclei preparation, but it does not necessarily invalidate the hypothesis that a G-protein involved in the signal transduction could be localized in the nuclei controlling phosphorylation/dephosphorylation of transcription factors or other proteins. Other explanations are certainly possible.

As mentioned earlier, the 75 kDa protein was not found in crude extract from etiolated *Avena* seedlings, whereas the 24 kDa protein was the major immunodetectable G-protein in the crude extract [19]. Thus, the weak band at 24 kDa shown in Fig. 2 may have originated from a cytosolic/plasma membrane contamination in the nuclear preparation. Furthermore, the phosphorylation data shown in Fig. 1 are markedly different from those obtained from etiolated *Avena* crude extracts [21].

In conclusion, we suggest that phosphorylation/dephosphorylation of certain proteins in etiolated *Avena* nuclei may be regulated by G-proteins involved in signal transducing cascades. It should be emphasized that the discussion described herein is only suggestive and speculative. Clearly, further study is warranted to determine whether or not the observed results are relevant to the mechanism of phytochrome action and the involvement of G-proteins.

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