were found in Jasper County, 17 of which involved radio telemetry–tagged hens from Minnesota and Kansas. To determine clutch size, fertility, and egg success, we used only nests with eggs or egg shells in good condition at the time of discovery and not those known to be partially or fully depredated. Searches were timed so that about 90% of nests were hatched, depredated, or abandoned upon discovery; about 10% were still active when found. Although researcher disturbance of 47 active nests was suspect in biasing egg success rates, a separate study of this possibility was inconclusive (*7*). Hence, we tested rates of egg success with and without the disturbed clutches. When only undisturbed nests were used the test statistic was still highly significant (ϕ = 2.95; P = 0.0016). Fertility and egg success were calculated by dividing the number of fertile (germinal discs or embryos evident but eggs not always hatched or fully incubated) or hatched eggs by the total number of eggs in unparasitized, fully incubated clutches. We excluded 38 successful nests parasitized by ring-necked pheasants (*Phasianus colchicus*) in calculating egg success to avoid bias of low success in parasitized nests [R. L. Westemeier, J. E. Buhnerkempe, W. R. Edwards, J. D. Brawn, S. A. Simpson, *J. Wildl. Manage.* **62**, 854 (1998)]. All tests (1963–1991 data) in Fig. 2 and for egg fertility were based on a nonparametric test for trend developed by E. L. Lehmann [*Nonparametrics—Statistical Methods Based on Ranks* (McGraw-Hill, New York, 1975)] using the normal approximation in all cases.

25. R. E. Yeatter, *Ill. Nat. Hist. Surv. Bull.* **22**, 377 (1943).
26. J. Lockart, *Ill. Dep. Conserv. Tech. Bull.* (1968).
27. A. O. Gross, *Wis. Conserv. Comm. Bull.* (1930); F. N. Hamerstrom Jr., *Wilson Bull.* **51**, 105 (1939); C. W. Schwartz, *Univ. Mo. Stud.* **20**, 1 (1945); W. B. Grange, *Wis. Conserv. Dep. Publ.* **328** (1948); M. F. Baker, *Univ. Kans. Mus. Nat. Hist. Biol. Surv. Kans.* **5** (1953); F. L. Arthaud, thesis, University of Missouri (1968); N. J. Silvy, thesis, Kansas State University (1968); R. D. Drobney, thesis, University of Missouri (1973); L. H. Sisson, *The Sharp-Tailed Grouse in Nebraska, a Research Study* (Nebraska Game and Parks Commission, Lincoln, 1976); W. D. Svedarsky, dissertation, University of North Dakota (1979); L. A. Rice and A. V. Carter, *Completion Report No. 84-11* (South Dakota Department of Game, Fish, and Parks, Pierre, SD, 1982); G. J. Horak, *Kansas Fish and Game Commission Wildlife Bulletin No. 3* (1985); J. A. Newell, thesis, Montana State University (1987); D. P. Jones, thesis, University of Missouri (1988); J. E. Toepfer, dissertation, Montana State University (1988).
28. L. L. McDaniel, unpublished material.
29. Our nest data were highly representative of the focal population because, on average during 1963–1991, total nests found represented 46% (range, 28% to 76%) and 77% (range, 39% to 188%) of the number of males and hens, respectively, censused on booming grounds in Jasper County.
30. J. L. Bouzat, H. A. Lewin, K. N. Paige, *Am. Nat.* **152**, 1 (1998); J. L. Bouzat *et al.*, *Conserv. Biol.* **10**, 836 (1998).
31. Estimates of genetic variability were based on highly polymorphic type II markers (microsatellites), which are more sensitive for detecting potential changes in genetic diversity. The relatively small reduction in Illinois average heterozygosity (about 9%) results from the large numbers of alleles detected at the microsatellite loci. Estimates of genetic diversity based on type I markers (that is, markers associated with coding sequences of structural genes) would likely reveal a more drastic decline in average heterozygosity. The observed declines of fitness components in the Illinois population should not be attributed to the particular loci analyzed (which are noncoding and presumably neutral DNA sequences). The effects of inbreeding are probably more directly related to the expression of lethal recessive alleles at structural genes. Our results also indicate that allelic diversity is more drastically affected than heterozygosity, as is expected following population bottlenecks (M. Nei, T. Maruyama, R. Chakraborty, *Evolution* **29**, 1 1975). Declines in fitness in the Illinois population probably result from a drastic decrease in heterozygosity at structural genes and the random extinction of critical alleles through the population bottleneck.
32. Illinois State Water Survey, *Ill. Agric. Stat. Serv.* (1993); *ibid.* (1994); *ibid.* (1997).
33. Factors other than inbreeding can cause fertility and hatchability problems. Other possible factors included competing species that may transmit disease (pheasants and waterfowl), pollutants (oil and pesticides), and human disturbances. However, egg success was declining about one decade before there was a large abundance of pheasants in the mid-1980s, researcher disturbance of active nests (mostly the mid-1980s to 1991) (*7*), a large increase in oil production (1983), and pesticides used for no-till farming. Similarly, waterfowl, mostly mallards (*Anas platyrhynchos*), did not feed or nest with prairie chickens until the late 1970s. Also, hatch rates of northern bobwhites (*Colinus virginianus*), pheasants, and other sympatric species in the study area have not declined.
34. M. E. Gilpin and M. E. Soule, in *Conservation Biology: The Science of Scarcity and Diversity*, M. E. Soule, Ed. (Sinauer Associates, Sunderland, MA, 1986), pp. 19–34.
35. A. O. Gross, *Mem. Boston Soc. Nat. Hist.* **6**, 491 (1928).
36. E. A. Osterndorff, thesis, Texas A&M University (1995).
37. We thank the many field assistants who made this report possible and especially project assistants D. R. Vance and J. E. Buhnerkempe; R. J. Ellis led the project during 1963–1965. We appreciate technical reviews of the manuscript by K. M. Giesen, C. A. Phillips, and P. W. Brown and editing and figure graphics by T. E. Rice. J. E. Toepfer provided guidance in all phases of translocation efforts and, with P. S. Beringer, helped in securing birds from Minnesota for translocation to Illinois. Illinois wild turkeys were traded for Minnesota prairie chickens with the assistance of the Illinois Department of Natural Resources–Division of Wildlife Resources. Various other wildlife staff in Minnesota, North Dakota, Kansas, and Nebraska supported translocation of prairie chickens. L. L. McDaniel provided unpublished egg success data from the Valentine National Wildlife Refuge, Nebraska. This is a contribution in part of Federal Aid Wildlife Restoration Project W-66-R, with the Illinois Department of Natural Resources–Division of Natural Heritage, U. S. Fish & Wildlife Service, Illinois Natural History Survey, The Nature Conservancy, Illinois Nature Preserves Commission, Illinois Endangered Species Protection Board, and the University of Illinois at Urbana–Champaign; cooperating. Funding for translocations of prairie chickens was provided by the Illinois Wildlife Preservation Fund and Natural Areas Acquisition Fund. Funding for locating nests in 1997 was provided by the Illinois Council on Food and Agricultural Research (C-FAR).

10 August 1998; accepted 9 October 1998

Arabidopsis NPH1: A Flavoprotein with the Properties of a Photoreceptor for Phototropism

John M. Christie, Philippe Reymond, Gary K. Powell, Paul Bernasconi, Andrei A. Raibekas, Emmanuel Liscum, Winslow R. Briggs*

The *NPH1* gene of *Arabidopsis thaliana* encodes a 120-kilodalton serine-threonine protein kinase hypothesized to function as a photoreceptor for phototropism. When expressed in insect cells, the NPH1 protein is phosphorylated in response to blue light irradiation. The biochemical and photochemical properties of the photosensitive protein reflect those of the native protein in microsomal membranes. Recombinant NPH1 noncovalently binds flavin mononucleotide, a likely chromophore for light-dependent autophosphorylation. The fluorescence excitation spectrum of the recombinant protein is similar to the action spectrum for phototropism, consistent with the conclusion that NPH1 is an autophosphorylating flavoprotein photoreceptor mediating phototropic responses in higher plants.

Plants rely heavily on the surrounding light environment to regulate normal growth and development. Over the past two decades, considerable progress has been made in characterizing the phytochrome family of photoreceptors that monitor the red and far-red regions of the electromagnetic spectrum (*1*). However, only recently have advances been made that increase our understanding of ultraviolet-A (UV-A)–blue light perception in plants (*2*).

Cryptochromes are UV-A–blue light photoreceptors with homology to microbial DNA photolyases (*2*). Like photolyases, the cryptochromes contain dual light-harvesting chromophores—flavin adenine dinucleotide (FAD) and either a deazaflavin (*3*) or a pterin (*4*)—but exhibit no DNA repair activity (*4, 5*). The two cryptochrome genes of *Arabidopsis*, *CRY1* and *CRY2*, encode homologous proteins (*3, 6*) that appear to overlap in function

J. M. Christie and W. R. Briggs, Department of Plant Biology, Carnegie Institution of Washington, 260 Panama Street, Stanford, CA 94305, USA. P. Reymond, Institut de Biologie et de Physiologie Végétales, Université de Lausanne, 1015 Lausanne, Switzerland. G. K. Powell and P. Bernasconi, Novartis Agribusiness Biotechnology Research Inc., 3054 Cornwallis Road, Research Triangle Park, NC 27709, USA. A. A. Raibekas, Department of Biological Chemistry, University of Michigan, M5416 Medical Science I, Ann Arbor, MI 48109, USA. E. Liscum, Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA.

*To whom correspondence should be addressed. E-mail: briggs@andrew.stanford.edu

to mediate the blue light regulation of seedling development (*7*). CRY2 also plays a major role in floral induction (*8*).

Arabidopsis mutants deficient in phototropism, designated *nph1* (nonphototropic hypocotyl 1), were previously shown to lack the blue light–dependent phosphorylation of a 120-kD protein associated with the plasma membrane (*9*). Because mutants at the *NPH1* locus lack all known phototropic responses in *Arabidopsis*, it has been hypothesized that *NPH1* encodes a photoreceptor for phototropism (*9*). The *NPH1* gene was recently isolated and found to encode a serine-threonine protein kinase (*10*). The NH_2-terminal region of the NPH1 protein contains two copies of a motif, designated the LOV domain, present in a number of proteins from organisms including archaea, eubacteria, and eukaryotes. These include NIFL (*11*) and Aer (*12*), both of which are reported to bind FAD. The LOV domain has therefore been proposed to reflect a flavin-binding site, regulating kinase activity in response to blue light–induced redox changes (*10*).

To characterize NPH1 in the absence of other plant proteins, we expressed the 120-kD phosphoprotein in insect cells transfected with recombinant baculovirus containing the *NPH1* coding sequence (*13*). Although most of the recombinant NPH1 expressed (designated BacNPH1) was insoluble, a small amount of the protein was found to be localized to the soluble fraction (Fig. 1A). The soluble protein produced was recognized by specific polyclonal NPH1 antisera (Fig. 1B). The high specificity of the antibody is demonstrated by the lack of NPH1 protein in membrane fractions isolated from the null mutant *nph1-5* (*10*). The heterologously produced protein is slightly higher in molecular weight (125 kD) than the native protein because of the presence of additional peptide sequences derived from the baculovirus expression vector (*13*). Ultracentrifugation and protein immunoblot analysis revealed that soluble BacNPH1 is not membrane associated. In contrast, NPH1 is associated with the plasma membrane upon isolation from *Arabidopsis* and several other plant species (*14*). Thus, although the nature of its association with the plant plasma membrane remains to be determined, this process does not appear to be operative in insect cells.

Photophysiological, genetic, and biochemical evidence suggests that NPH1 is a putative photoreceptor (*9, 15*) that undergoes blue light–dependent autophosphorylation (*14*). We therefore investigated whether NPH1 expressed in insect cells could be phosphorylated in response to blue light irradiation. Insect cells expressing NPH1 were grown in complete darkness and harvested under dim red light. Soluble protein samples were isolated and used for in vitro phosphorylation analysis. Autoradiography revealed that BacNPH1 is highly phosphorylated after a brief irradiation with blue light (Fig. 1C). No light-activated phosphorylation was detectable in soluble fractions prepared from control cells expressing biotin carboxylase. These results indicate that NPH1 is a photosensitive, autophosphorylating protein kinase. The blue light–induced phosphorylation of BacNPH1 was observed with six independently transfected cultures of insect cells. Furthermore, the relative increase in phosphorylation induced by blue light is comparable to that observed in membrane fractions isolated from etiolated *Arabidopsis* seedlings (Fig. 1C). Remarkably, both the fluence-response requirements and the phosphorylation kinetics of BacNPH1 strongly resemble those of the native protein in microsomal membranes (Fig. 2, A and B). In contrast, after a saturating light pulse, recovery of light sensitivity in darkness for native NPH1 is more rapid than that observed for the recombinant protein (Fig. 2C). Dark attenuation of the in vitro blue light–mediated phosphorylation has also been reported for pea (*16*), maize (*17*), and oat (*18*) and is believed to restore the light-sensitive phosphorylation system to its initial ground state. An explanation for the observed difference could be that some *Arabidopsis* protein or factor involved in desensitizing NPH1 activation in the absence of light is simply lacking from insect cells.

The above results support the earlier hypothesis (*9*) that NPH1 is a photoreceptor mediating blue light–dependent autophosphorylation. We therefore investigated whether BacNPH1 binds a cofactor that could function as a light-harvesting chromophore. Several chromophore moieties have been proposed for blue light photoreceptors, including carotene (*19*), flavins (*20*), pterins (*21*), reti-

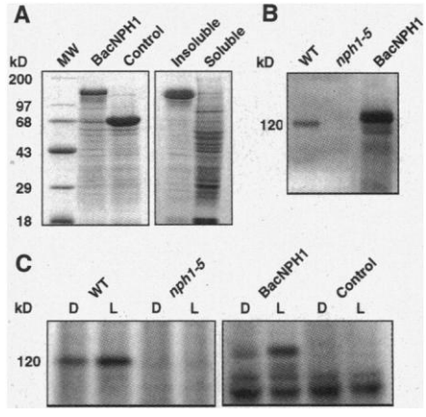

Fig. 1. (**A**) Expression of NPH1 in insect cells. A Coomassie blue–stained SDS-polyacrylamide gel (12.5%) is shown for the total protein of insect cells expressing NPH1 (BacNPH1) or biotin carboxylase (control). Also shown are the proteins of pelleted (insoluble) and soluble fractions isolated from insect cells expressing NPH1. Cells were lysed by sonication and separated into pelleted and soluble fractions by centrifugation at 16,000*g* for 10 min. Molecular weight markers (MW) are shown on the left. (**B**) Immunoblot analysis of NPH1 protein. Growth of Columbia wild-type (WT) and *nph1-5* mutant seedlings and preparation of microsomal membranes were as described (*9*). Membrane protein (20 μg) and soluble protein (5 μg) prepared from insect cells expressing NPH1 (BacNPH1) were resolved on an SDS-polyacrylamide gel (7.5%), and the immunoblot was probed with anti-NPH1 (*30*). (**C**) Autoradiogram showing the blue light–dependent phosphorylation of BacNPH1. Membrane preparations from etiolated wild-type (WT) or *nph1-5* mutant seedlings and soluble protein extracts isolated from insect cells expressing NPH1 (BacNPH1) or biotin carboxylase (control) were used for in vitro phosphorylation analysis, as described (*9*). All manipulations were carried out under dim red light. Samples were given a mock irradiation (D) or irradiated with blue light (L) at a total fluence of 3300 μmol m^{-2}.

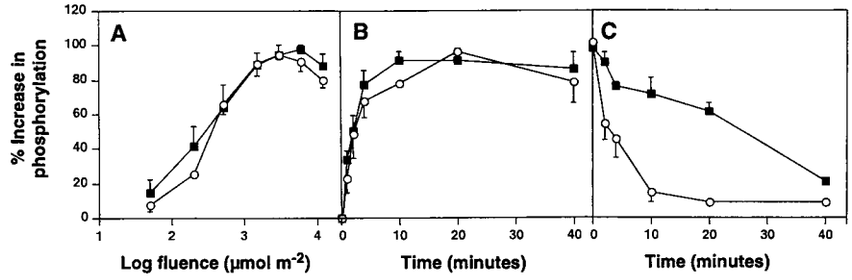

Fig. 2. (**A**) Fluence response of NPH1 phosphorylation in wild-type membranes (○) and soluble protein extracts prepared from insect cells expressing NPH1 (■). (**B**) Kinetics of NPH1 phosphorylation in wild-type membranes (○) and soluble protein extracts prepared from insect cells expressing NPH1 (■). (**C**) Effect of dark incubation on NPH1 phosphorylation in wild-type membranes (○) and soluble protein extracts prepared from insect cells expressing NPH1 (■). Samples were irradiated and incubated on ice for the times indicated before the addition of radiolabeled adenosine triphosphate. In each case, all values are relative to dark controls and represent the average of three independent experiments. Standard errors are shown. The extent of phosphorylation was quantified with a PhosphorImager (Molecular Dynamics).

nal (*22*), and zeaxanthin (*23*). Because the insoluble form of BacNPH1 represents the majority of extractable protein from insect cells (Fig. 1A), this fraction was initially used for the analysis of potential chromophores. The cofactor was found to be noncovalently bound, as it was released by heat or acid denaturation of BacNPH1. Spectral analysis of the released chromophore revealed the pigment to be fluorescent, with excitation and emission maxima resembling those of free flavins (Fig. 3, A and B). Furthermore, the absorption spectrum of the free chromophore exhibited the two prominent absorbance peaks characteristic of flavins (Fig. 3C). These spectral characteristics were also detectable in soluble protein extracts prepared from insect cells expressing BacNPH1 but were minimal in extracts from control cells expressing biotin carboxylase (Fig. 3, A to C). (A background level of flavin fluorescence was routinely observed in soluble fractions isolated from the control cells, likely from flavins released from endogenous flavoproteins present.) The flavin associated with BacNPH1 was identified as flavin mononucleotide (FMN) by thin-layer chromatography, according to its mobility relative to FAD, FMN, and riboflavin standards (Fig. 3D). These observations confirm previous biochemical evidence suggesting that the photodetection mechanism for the blue light–dependent phosphorylation reaction requires a flavin species (*24*).

Phototropism is induced by green light in addition to UV-A–blue light in *Arabidopsis* (*9, 25*). It will be interesting to establish whether the redox properties of the FMN bound to NPH1 lead to a stable semiquinone, thereby generating additional sensitivity in the green region of the spectrum, as was found for the FAD chromophore bound to CRY1 (*5*). Given that the fluence-response requirements and phosphorylation kinetics for BacNPH1 correspond to those of the native protein in microsomal membranes (Fig. 2, A and B), it seems most likely that FMN is also the chromophore that mediates the photoactivation of NPH1 in vivo.

Action spectra for a number of processes initiated by UV-A–blue light, including phototropism, have been described (*26*) and are reported to resemble the absorption spectrum of a flavoprotein. The action spectrum for phototropism shows maximal activity between 400 and 500 nm and reveals a degree of fine structure with a major band at 450 nm and subsidiary shoulders at 430 and 470 nm (dashed line, Fig. 4B). An additional broad, less effective peak is typically observed at 380 nm. We therefore examined whether a similar degree of fine structure could be detected for the insoluble form of BacNPH1. Indeed, the uncorrected fluorescence excitation spectrum for BacNPH1 displays the characteristic fine structure observed in the action spectrum for phototropism (Fig. 4A). Such fluorescent peaks were also clearly visible in soluble protein extracts prepared from insect cells expressing NPH1, but they were undetectable in extracts from cells expressing biotin carboxylase (Fig. 4A). Subtraction of the background fluorescence observed in the control gave a corrected fluorescence excitation spectrum for BacNPH1 that resembles the action spectrum for phototropism (Fig. 4B). The turbid nature of the sample used for this analysis would be expected to reduce fluorescence in the UV-A region of the spectrum and to account for the less prominent peak at 380 nm. These findings are also consistent with the hypothesis that *NPH1* encodes the apoprotein of a blue light photoreceptor for phototropism.

Recent genetic evidence has implicated an involvement of cryptochrome in phototropism (*27*). An *Arabidopsis* mutant lacking CRY1 and functional CRY2 displayed an apparent lack of first-positive phototropic curvature in response to blue light irradiation. However, *cry1cry2* double mutants retained a significant degree of second-positive curvature, indicating the presence of an independent photoreception system for phototropism. Null mutants lacking CRY1 and CRY2 protein exhibited first-positive blue light–induced phototropic curvature (*28*), suggesting that CRY1 and CRY2 are not the primary photoreceptors mediating phototropic curvature in *Arabidopsis*. The *cry1cry2* double mutants also retained normal in vitro blue light–dependent phosphorylation of NPH1 (*28*). Thus, light-induced phosphorylation of NPH1 does not appear to result from the action of cryptochrome. Instead, it is possible that cryptochrome, like phytochrome (*29*), functions to modulate the response output, leading to enhanced first- and second-positive phototropic curvatures. A more detailed photophysiological characterization of phototropic responses in cryptochrome- and phytochrome-deficient mutants will aid our understanding of the phototropic detection system, which appears to involve the interaction of both red–far-red and other blue light photoreceptors. The present results, in conjunc-

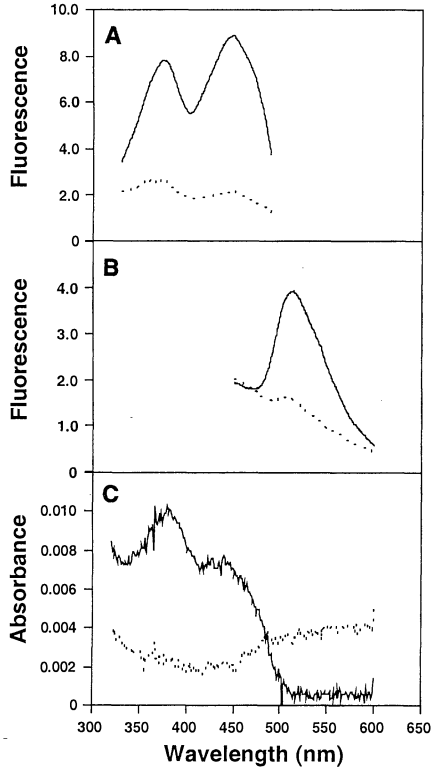

Fig. 3. (**A** to **C**) The fluorescence excitation spectrum (A), fluorescence emission spectrum (B), and absorption spectrum (C) of the chromophore released from insoluble protein extracts prepared from insect cells expressing NPH1 (solid lines). In each case, an equal amount of protein extract from insect cells expressing biotin carboxylase (dotted line) was used as a control (*31*). (**D**) Identification of the chromophore bound to BacNPH1 as FMN. The chromophore bound to BacNPH1 was released by boiling the pelleted fraction (1 mg) in 70% ethanol and used for thin-layer chromatography as described (*32*) using *n*-butanol–acetic acid–water (3:1:1 v/v) as solvent. Retardation factor (R_f) values for the BacNPH1 chromophore (BacNPH1C) and other flavins are shown.

D

Flavin	*Rf*
BacNPH1C	0.31
Riboflavin	0.63
FMN	0.31
FAD	0.18

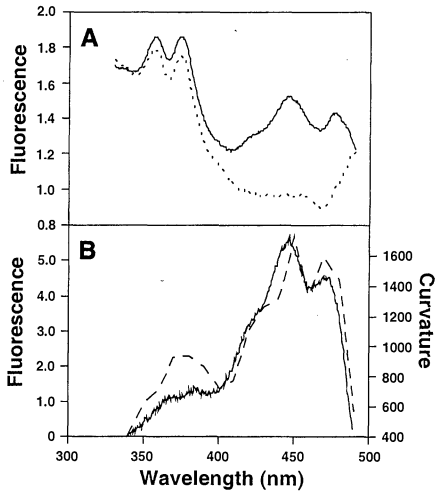

Fig. 4. (**A**) Fluorescence excitation spectra of insoluble protein extracts prepared from insect cells expressing NPH1 (solid line) and biotin carboxylase (dotted line). (**B**) The corrected fluorescence excitation spectrum (solid line) for BacNPH1 [the difference between the fluorescence spectra shown in (A)] plotted with the action spectrum for the ascending arm of alfalfa hypocotyl phototropism [dashed line; redrawn from (*33*)].

tion with the established role of NPH1 in phototropism (*9, 10*), lead us to propose that NPH1 is an autophosphorylating flavoprotein, unrelated to cryptochrome, that serves as a photoreceptor for phototropism in higher plants.

References and Notes

1. P. H. Quail *et al.*, *Science* **268**, 675 (1995); P. Quail, *Bioessays* **19**, 571 (1997).
2. M. Ahmad and A. R. Cashmore, *Plant Mol. Biol.* **30**, 851 (1996).
3. ———, *Nature* **366**, 162 (1993).
4. K. Malhotra *et al.*, *Biochemistry* **34**, 6892 (1995).
5. C. Lin *et al.*, *Science* **269**, 968 (1995).
6. C. Lin, M. Ahmad, J. Chan, A. R. Cashmore, *Plant Physiol.* **110**, 1047 (1996).
7. M. Ahmad, J. A. Jarillo, A. R. Cashmore, *Plant Cell* **10**, 197 (1998); C. Lin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2686 (1998).
8. H. Guo, H. Yang, T. C. Mockler, C. Lin, *Science* **279**, 1360 (1998).
9. E. Liscum and W. R. Briggs, *Plant Cell* **7**, 473 (1995).
10. E. Huala *et al.*, *Science* **278**, 2120 (1997).
11. S. Hill, S. Austin, T. Eydmann, T. Jones, R. Dixon, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2143 (1996).
12. S. I. Bibikov, R. Biran, K. E. Rudd, J. S. Parkinson, *J. Bacteriol.* **179**, 4075 (1997).
13. The coding sequence of *NPH1* was inserted at the Eco RI site after the polyhistidine tag and the thrombin cleavage site of the baculovirus transfer vector pAcHLT-A (Pharmingen) and transfected into Sf9 (*Spodoptera frugiperda*) insect cells (American Type Culture Collection) in accordance with Pharmingen's instructions. Recombinant virus was titered by end-point dilution and used to infect *Trichoplusia ni* insect cells (High-Five, Invitrogen). Cells were grown in serum-free medium (Excell 400; JRH Biosciences, Lenexa, KS) at 26°C in light-tight culture flasks (150 cm^2). After 4 days, cells were harvested by centrifugation at 1000*g* for 2 min and resuspended in phosphorylation buffer (9). Throughout the study, protein fractions prepared from insect cells expressing the biotin carboxylase apoprotein from *Ustilago maydis* were used as a control.
14. P. Reymond, T. W. Short, W. R. Briggs, *Plant Physiol.* **100**, 655 (1992).
15. T. W. Short and W. R. Briggs, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 143 (1994); W. R. Briggs and E. Liscum, in *Signal Transduction in Plants*, P. Aducci, Ed. (Birkhäuser, Basel, Switzerland, 1997), p. 107.
16. T. W. Short and W. R. Briggs, *Plant Physiol.* **92**, 179 (1990).
17. J. M. Palmer, T. W. Short, S. Gallagher, W. R. Briggs, *ibid.* **102**, 1211 (1993).
18. M. Salomon, M. Zacherl, L. Luff, W. Rüdiger, *ibid.* **115**, 493 (1997).
19. G. Wald and H. G. DuBuy, *Science* **84**, 247 (1936).
20. A. W. Galston, in *Handbuch der Planzenphysiologie*, W. Ruhland, Ed. (Springer-Verlag, Berlin, 1949), vol. XVII, part 1, p. 492.
21. P. Galland and H. Senger, *Photochem. Photobiol.* **48**, 811 (1988).
22. R. Lorenzi, N. Ceccarellin, B. Lercari, P. Guattieri, *Phytochemistry* **36**, 599 (1994).
23. E. Zeiger and J. Zhu, *J. Exp. Bot.* **49**, 433 (1998).
24. T. W. Short, M. Porst, W. R. Briggs, *Photochem. Photobiol.* **55**, 773 (1992).
25. R. Konjevic, B. Steinitz, K. L. Poff, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9876 (1989).
26. W. R. Briggs and M. Iino, *Philos. Trans. R. Soc. London Ser. B* **303**, 347 (1983).
27. M. Ahmad, J. A. Jarillo, O. Smirnova, A. R. Cashmore, *Nature* **392**, 720 (1998).
28. Using a fluence of $10^{0.5}$ μmol m^{-2}, we obtained 15.5 ± 2.6, 6.6 ± 0.9, and 8.8 ± 1.4 degrees of hypocotyl curvature for 3-day-old etiolated seedlings of the wild type (Columbia), the double mutant *hy4-B104(cry1)cry2-1*, and the double mutant *cry1-304cry2-1*, respectively. With 2-day-old etiolated seedlings, we observed negligible curvature for the wild type, 12.0 ± 1.1 degrees for the double mutant *hy4-B104(cry1)cry2-1*, and 14.8 ± 1.8 degrees for the double mutant *cry1-304cry2-1*. Both *cry1cry2* mutants exhibited normal phosphorylation in response to a saturating pulse of blue light ($10^{3.3}$ μmol m^{-2}) (M. A. Olney, J. M. Christie, W. R. Briggs, unpublished data).
29. A. K. Janoudi, R. Konjevic, G. Whitelam, W. Gordon, K. L. Poff, *Physiol. Plant.* **101**, 278 (1997).
30. Antibody to NPH1 was produced by cloning the NH_2-terminal third of *NPH1* including the LOV1 domain, designated *NPH1N*, to the Eco RI–Pst I site of the vector pMal-c2 (New England Biolabs) as a translational fusion to the maltose binding protein (MBP). MBP-NPH1N was expressed in *Escherichia coli*, purified by amylose-affinity chromatography, and used to prepare polyclonal antibodies in rabbits (Cocalico Biologicals, Reamstown, PA). Immunoblots were analyzed with anti-NPH1 (1:1000 dilution) using the color development method (Promega) with antibody to rabbit immunoglobulin G conjugated to alkaline phosphatase as the secondary antibody.
31. Spectroscopic analysis was performed with a Beckman DU-70 spectrophotometer and a Photon Technology International Alphascan spectrofluorometer. Fluorescence excitation spectra were obtained by monitoring the emission at 535 nm. Fluorescence emission spectra were measured by using an excitation wavelength of 390 nm. For fluorescence measurements, insoluble fractions (1 mg) were treated with 6 M guanidine-HCl, 0.1 M sodium phosphate, and 0.01 M tris-HCl (pH 8.0). For absorption studies, insoluble fractions were treated with 10% trichloroacetic acid. In each case, samples were centrifuged at 16,000*g* for 10 min and the supernatants were analyzed for the presence of chromophore.
32. A. A. Raibekas, *J. Biolumin. Chemilumin.* **6**, 169 (1991).
33. T. I. Baskin and M. Iino, *Photochem. Photobiol.* **46**, 127 (1987).

34. We thank T. Baskin for providing the phototropism action spectrum data, and M. A. Olney and E. Huala for their assistance and critical reading of the manuscript. P.R. thanks the Fondation du 450ue Anniversaire of the University of Lausanne for providing a travel grant. Supported by NSF grants IBN-9219256 and IBN-960114. This paper is Carnegie Institution of Washington, Department of Plant Biology publication 1385.

24 August 1998; accepted 23 October 1998

Purification and Cloning of a Protein Kinase That Phosphorylates and Activates the Polo-Like Kinase Plx1

Yue-Wei Qian, Eleanor Erikson, James L. Maller*

The *Xenopus* polo-like kinase 1 (Plx1) is essential during mitosis for the activation of Cdc25C, for spindle assembly, and for cyclin B degradation. Polo-like kinases from various organisms are activated by phosphorylation by an unidentified protein kinase. A protein kinase, polo-like kinase kinase 1 or xPlkk1, that phosphorylates and activates Plx1 in vitro was purified to near homogeneity and cloned. Phosphopeptide mapping of Plx1 phosphorylated in vitro by recombinant xPlkk1 or in progesterone-treated oocytes indicates that xPlkk1 may activate Plx1 in vivo. The xPlkk1 protein itself was also activated by phosphorylation on serine and threonine residues, and the kinetics of activation of xPlkk1 in vivo closely paralleled the activation of Plx1. Moreover, microinjection of xPlkk1 into *Xenopus* oocytes accelerated the timing of activation of Plx1 and the transition from G_2 to M phase of the cell cycle. These results define a protein kinase cascade that regulates several events of mitosis.

Progression through the eukaryotic cell cycle relies on the periodic activation or inactivation of various cyclin-dependent protein kinases (Cdks) (*1*). Cell cycle checkpoints monitor the fidelity of events in a given cell cycle phase and control a signaling system that can delay cell cycle progression and changes in Cdk activity. One checkpoint blocks activation of the Cdc2–cyclin B complex in G_2 phase if DNA replication is incomplete. This block to activation is mainly accomplished by maintenance of the phosphorylated state of Thr^{14} and Tyr^{15} in Cdc2 (*2*). These considerations have focused attention on the pathway of activation of the phosphatase Cdc25C, which initiates mitotic entry by dephosphorylating Thr^{14} and Tyr^{15} in Cdc2 (*3*). Overexpression of Cdc25C either in vivo (*4*) or in vitro (*5, 6*) overcomes the replication checkpoint. Cdc25C is activated at the G_2-M transition by serine-threonine phosphorylation (*6, 7*). The Cdc2–cyclin B complex can phosphorylate and activate Cdc25C (*8*), forming a positive feedback loop, but in *Xenopus* initial phosphorylation and activation of Cdc25C results from activation of the polo-like kinase Plx1 (*9, 10*). Plx1 can phosphorylate Cdc25C in vitro at activating sites (*9*), and in vivo activation of Plx1 coincides with the activation of Cdc25C (*10*). Moreover, inhibition of Plx1 delays the activation of Cdc25C, and microinjection of

Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262, USA.

*To whom correspondence should be addressed. E-mail: mallerj@essex.uchsc.edu