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13. Flies were reared in 12-hour cycles of light and darkness (LD 12:12) at 25°C with lights on at 9 a.m. and off at 9 p.m. Males 2 to 7 days old were placed in the locomotor activity tubes at either 18°C or 29°C and exposed to two additional LD cycles, after which data collection in constant darkness (DD) was initiated. Locomotor activity data was collected in 30-min "time windows" for 7 days. Autocorrelation and spectral analysis were carried out on each male's activity record (*14, 15*).
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17. Two *(Thr-Gly)20* and two Δ*(Thr-Gly)* transformant lines in which the *per* transgenes were inserted within the cp20.1 vector marked with the *rosy*$^+$ gene were used (*18*). In addition, the *(Thr-Gly)20 per* transgene was also ligated into the pW8 vector marked with *white*$^+$ to further produce two transformant lines. The *(Thr-Gly)*1 construct was made by amplifying a 716–base pair (bp) fragment from the *(Thr-Gly)*20 clone and incorporating a deletion encoding 19 Thr-Gly pairs. This was done with 5′ primer (A), 5′-AACTATAACGAGAACCTGCT-3′ (4874 to 4893); with 3′ primer (B), 5′-ATTGCCGGTACCAC-CAGTGCCGGCAATGCT-3′ (5094 to 5113); with 5′ primer (C), 5′-GCACTGGTGGTACCGGCAATG-GAACAAATTCCGG-3′ (5231 to 5250); and with 3′ primer (D), 5′-GCTACGCCTGTTCCGGATCC-3′ (5627 to 5646), using hybrid polymerase chain reaction (PCR) methods (*25*). Numbers in brackets denote the nucleotide positions corresponding to the *per* sequence described (*26*). Primer B and C create a Kpn I site (GGTACC) using the two underlined mismatch bases. Primer A ends 37 bp upstream of an Sst I site, and primer D incorporates a Bam HI site (underlined); these sites were used to substitute a 607-bp Thr-Gly deleted fragment into a 7-kb Xba fragment, which encodes the 3′ half of the *per* gene. This Xba fragment was then ligated to a 5′ Bam HI–Xba fragment in several cloning steps, thereby reconstituting the 13.2-kb *per* transcription unit within the pW8 transformation vector. The *(Thr-Gly)17* construct was generated by amplifying a 364-bp Thr-Gly fragment using a natural *(Thr-Gly)17a* variant as template and 5′ primer (A), in conjunction with 3′ primer 5′-CATTGCCGGTACCAGTGCCT-3′ (5199 to 5215 and 5233 to 5236), which carried two mismatch bases (underlined) forming a Kpn I site. The amplified product was digested with Sst I (cuts at position 4930) and Kpn I, and was used to replace the Sst I–Kpn I fragment in the *(Thr-Gly)*1 construct.
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19. The mean free-running spectrally derived periods of the two Δ*(Thr-Gly)* transformant lines were 25.02 and 24.58 hours at 18°C, and 26.16 and 27.38 hours at 29°C, respectively; those for the three *(Thr-Gly)1* lines were 24.76, 24.34, and 24.93 hours at 18°C, and 26.05, 26.25, and 26.58 hours at 29°C, respectively; for the two *(Thr-Gly)17* lines, periods were 23.4 and 24.3 hours at 18°C, and 24.27 and 25.48 hours at 29°C, respectively; periods for the four *(Thr-Gly)20* lines were 24.61, 24.07, 24.21, and 23.51 hours at 18°C, and 24.80, 24.18, 24.92, and 25.43 hours at 29°C, respectively. Periods derived from autocorrelation were almost identical.
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28. L. A. Sawyer *et al.*, unpublished results.

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Arabidopsis NPH1: A Protein Kinase with a Putative Redox-Sensing Domain

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The *NPH1* (nonphototropic hypocotyl 1) gene encodes an essential component acting very early in the signal-transduction chain for phototropism. *Arabidopsis NPH1* contains a serine-threonine kinase domain and LOV1 and LOV2 repeats that share similarity (36 to 56 percent) with *Halobacterium salinarium* Bat, *Azotobacter vinelandii* NIFL, *Neurospora crassa* White Collar–1, *Escherichia coli* Aer, and the Eag family of potassium-channel proteins from *Drosophila* and mammals. Sequence similarity with a known (NIFL) and a suspected (Aer) flavoprotein suggests that NPH1 LOV1 and LOV2 may be flavin-binding domains that regulate kinase activity in response to blue light–induced redox changes.

Plants irradiated with unilateral blue light grow toward the light source, a phenomenon known as phototropism. We studied a 120-kD plant plasma membrane–associated protein that becomes heavily phosphorylated on irradiation with blue light both in vivo and in vitro. Physiological and genetic evidence implicates this protein in an early step in the signal-transduction pathway for phototropism (*1*, *2*). We previously isolated phototropism mutants at four loci in *Arabidopsis thaliana* (L.) Heynh., designated *NPH1* through *NPH4* for nonphototropic hypocotyl (*2*). Three *nph1* alleles generated by fast neutron irradiation (*nph1-1*, *nph1-4*, and *nph1-5*) lack all known phototropic responses, demonstrating that the NPH1 protein is essential for all phototropic signal-transduction pathways in *Arabidopsis*. In addition, these mutants lack the 120-kD phosphoprotein, suggesting that *NPH1* might encode the phosphoprotein itself (*2*).

The *NPH1* locus is located on chromosome III, within 26 centimorgans (cM) of *GL1* (*2*). Using described methods (*3*), we employed amplified fragment length polymorphism (AFLP) (*4*) to identify DNA markers flanking the *NPH1* gene at distances of 0.3 and 0.4 cM (Fig. 1A). These markers were used to screen an *Arabidopsis* yeast artificial chromosome (YAC) library (*5*) by polymerase chain reaction (PCR) (*6*). Of three YACs identified, two (yUP1C7 and yUP21F3) overlap each other and map to chromosome III near the *NPH1* gene (*2*). Using sequences from yUP1C7 as a probe, we detected an altered restriction fragment pattern in *nph1-5* and cloned an 18-kb Eco RI fragment that appeared in *nph1-5* but not in the wild type. From this fragment, we subcloned a 7-kb Bgl II fragment that contains an end point of the DNA rearrangement in this mutant (Fig. 1B). Because we had biochemical evidence that the *NPH1* gene product was

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likely to be a 120-kD protein (*2*), we used a variety of probes from the region surrounding the breakpoint in *nph1-5* to look for an mRNA of the appropriate size (about 3.3 kb) on a northern blot of wild-type RNA. We detected a message of 3.35 kb (Fig. 1C) and isolated its wild-type cDNA and genomic clones. Complementation of the *nph1-5* mutation with genomic clone p51 restored the mutant's phototropic response (Fig. 1, D to F).

We sequenced the cDNA and genomic clones of *NPH1* and found that the gene encodes a protein of 996 amino acids (112 kD) (Fig. 2A). The coding region consists of 20 exons extending for 5.4 kb (Fig. 2B). Analysis of the predicted amino acid sequence suggests that the COOH-terminal region of NPH1 encodes a serine-threonine protein kinase (Fig. 2A). The NPH1 kinase domain contains the 11 sequence motifs typical of protein kinases and falls into the PVPK1 family of serine-threonine protein kinases within the protein kinase C group (*7*). Previous experiments have shown that the 120-kD phosphoprotein from maize and pea is phosphorylated on serine and threonine residues and not on tyrosine residues (*8*) and is itself a kinase that mediates autophosphorylation (*9*).

We compared the deduced NPH1 protein sequence with GenBank sequences (*10*) and identified three sequences that are likely to be from COOH-terminal partial clones of *NPH1* homologs from pea (*PsPK5*) (*11*), spinach (*SK3*) (*12*), and ice plant (*MK10*) (*13*). These three sequences are 60 to 76% identical at the protein level with *Arabidopsis* NPH1 over their entire length, including sequences outside the kinase domain. Phy3 from the fern *Adiantum capillus-veneris* is also similar to NPH1 but, unlike NPH1, has a phytochrome-like domain at its NH_2-terminal end (*14*).

We also sequenced three mutant *nph1* alleles (Fig. 2A) (*15*). The ethylmethane sulfonate–induced *nph1-2* mutant (*16*) has a single base substitution near the end of the kinase domain, which results in the substitution of a lysine for an arginine at position 936 (*17*). This invariant arginine residue maintains the structure of the COOH-terminal lobe of the kinase domain by forming an ion pair with a glutamic acid residue at position 862 (*7*). Of the two fast neutron mutants sequenced, one (*nph1-5*) carries a large-scale rearrangement that disrupts the *NPH1* gene within an intron following position 565 of the predicted protein sequence. The other, *nph1-1* (*18*), carries a three-nucleotide deletion that removes a valine residue from position 774 in the kinase domain, within a highly conserved motif that forms an α helical structure (*7*). Together with the complementation of the mutant with a genomic clone, the presence of a lesion in each of the three *nph1* mutants we sequenced indicates that the gene we cloned is *NPH1*. The size of the *NPH1* gene, the presence of a serine-threonine protein kinase domain in the NPH1-deduced protein sequence, and the absence of the 120-kD protein from the *nph1* mutants all suggest that the *NPH1* gene encodes the 120-kD phosphoprotein. Although the

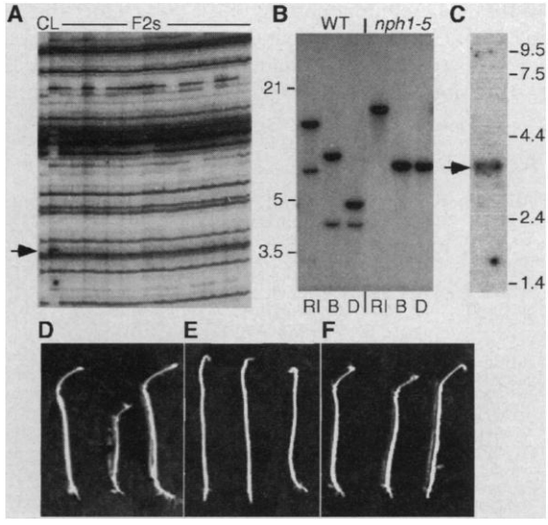

Fig. 1. Cloning of *NPH1*. (**A**) Autoradiogram of a typical AFLP gel. Lanes 1 and 2, wild-type Columbia (C) and Landsberg *erecta* (L) parental DNA; lanes 3 to 18, DNA from individual *nph1-1* homozygous F2 plants (F2s) derived from a Columbia *nph1-1/nph1-1* × Landsberg *erecta NPH1/NPH1* cross. The arrow denotes a Landsberg-specific band that shows tight linkage to *NPH1* (1 heterozygous recombinant in 258 homozygous *nph1-1/nph1-1* plants). The bands shown range in size from 120 to 220 base pairs. (**B**) Autoradiogram of a Southern blot of wild-type (WT) and *nph1-5* genomic DNA digested with Eco RI (RI), Bgl II (B), and both enzymes (D) and probed with a 7-kb Bgl II fragment subcloned from an 18-kb Eco RI fragment present in *nph1-5* but not in the wild type. The 7-kb Bgl II fragment contains an end point of the deletion and rearrangement in *nph1-5*. The positions of molecular size markers are shown in kilobases on the left. (**C**) Northern blot of wild-type mRNA probed with the 7-kb Bgl II fragment from *nph1-5*. The probe hybridized to a single band (arrow) corresponding to a message of ~3350 nucleotides. The positions of molecular size markers are shown in kilobases on the right. (**D** to **F**) Wild type (D), *nph1-5* (E), and *nph1-5* (F) transformed with genomic clone p51. Plants were transformed by vacuum infiltration with p51, a 17-kb genomic clone containing the *NPH1* gene. Seeds were vernalized, given 30 min of red light to stimulate germination, grown for 3 days in darkness, and treated with 16 hours of unilateral blue light from the right. The presence of both wild-type and mutant copies of *NPH1* in transformed plants was verified by Southern blot analysis (*25*).

Fig. 2. The *NPH1* coding region. (**A**) Predicted amino acid sequence of NPH1 (*26*). The sequence of amino acids 1 to 7 was obtained from a genomic clone, and amino acids 8 to 996 were obtained from a partial cDNA clone (GenBank accession number AF030864). The genomic clone was isolated from a library made from partially Hind III–digested *Arabidopsis* (Columbia) genomic DNA cloned into the cosmid pBIC20 (*27*), and the cDNA clone was isolated from a cDNA library made from size-selected 3-day-old *Arabidopsis* (Columbia) seedling hypocotyls (*28*). Amino acid residues are numbered on the right. The LOV1 and LOV2 domains are doubly underlined, and the kinase domain is singly underlined. The last amino acid residue before the breakpoint in *nph1-5* (position 566) is shaded in black. The amino acid residues affected in *nph1-1* and *nph1-2* (loss of valine 774 in *nph1-1* and substitution of lysine for arginine 936 in *nph1-2*) are also shaded in black. (**B**) Exon structure of *NPH1*. The restriction map of the region around *NPH1* is shown above (E, Eco RI; Bg, Bgl II; S, Sal I; B, Bam HI; and X, Xba I), and the position of the *NPH1* gene is shown below. Black boxes represent exons and lines represent introns.

A

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MEPTEKPSTKPSSRTLPRDTRGSLEVFNPSTQLTRPDNPVFRPEPPAWQN  50
LSDPRGTSPQPRPQQEPAPSNPVRSDQEIAVTTSWMALKDPSPETISKKT 100
ITAEKPQKSAVAAEQRAAEWGLVLKTDTKTGKPQGVGVRNSGGTENDPNG 150
KKTTSQRNSQNSCRSSGEMSDGDVPGGRSGIPRVSEDLKDALSTFQQTFV 200
VSDATKPDYPIMYASAGFFNMTGYTSKEVVGRNCRFLQGSGTDADELAKI 250
RETLAAGNNYCGRILNYKKDGTSFWNLLTIAPIKDESGKVLKFIGMQVEV 300
SKHTEGAKEKALRPNGLPESLIRYDARQKDMATNSVTELVEAVKRPRALS 350
ESTNLHPFMTKSESDELPKKPARRMSENVVPSGRRNSGGGRRNSMQRINE 400
IPEKKSRKSSLSFMGIKKKSESLDESIDDGFIEYGEEDDEISDRDERPES 450
VDDKVRQKEMRKGIDLATTLERIEKNFVITDPRLPDNPIIFASDSFLELT 500
EYSREEILGRNCRFLQGPETDLTTVKKIRNAIDNQTEVTVQLINYTKSGK 550
KFWNIFHLQPMRDQKGEVQYFIGVQLDGSKHVEPVRNVIEETAVKEGEDL 600
VKKTAVNIDEAVRELPDANMTPEDLWANHSKVVHCKPHRKDSPPWIAIQK 650
VLESGEPIGLKHFKPVKPLGSGDTGSVHLVELVGTDQLFAMKAMDKAVML 700
NRNKVHRARAEREILDLLDHPFLPALYASFQTKTHICLITDYYPGGELFM 750
LLDRQPRKVLKEDAVRFYAAQVVLALEYLHCQGIIYRDLKPENVLIQGNG 800
DISLSDFDLSCLTSCKPQLLIPSIDEKKKKKQQKSQQTPIFMAEPMRASN 850
SFVGTEEYIAPEIISGAGHTSAVDWWALGILMYEMLYGYTPFRGKTRQKT 900
FTNVLQKDLKFPASIPASLQVKQLIFRLLQRDPKKRLGCFEGANEVKQHS 950
FFKGINWALIRCTNPPELETPIFSGEAENGEKVVDPELEDLQTNVF     996
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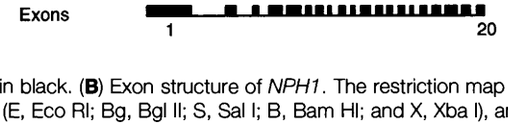
B



phosphoprotein is associated with the plasma membrane on isolation (*1*), a hydrophobicity analysis of NPH1 suggests that it is a soluble protein without membrane-spanning domains. The nature of its association with the plasma membrane remains to be determined.

In the NH_2-terminal portion of the predicted NPH1 protein, we found two 107–amino acid sequences that share 43% identity (61% similarity) (Fig. 2A). The motif of these repeats (LOV1 and LOV2) also appears as a single copy in proteins from archaea, eubacteria, and eukaryotes (Fig. 3A) that otherwise share no similarity with NPH1 and have diverse functions (Fig. 3B). However, those for which a function is known are regulated by environmental factors that could change their redox status: light, oxygen, or voltage (hence, LOV). For both NIFL (*19*) and Aer (*20*), the mechanism for redox sensing appears to be oxidation or reduction of a flavin prosthetic group. For voltage-gated potassium channels such as ELK, Eag, and ERG, voltage sensing is mediated by charged residues in the fourth transmembrane segment S4 (*21*). The NH_2-terminal part of the channel protein, including the LOV domain, participates in the formation of homotetrameric structures (*22*). Although no function in voltage sensing is currently attributed to the LOV domain in potassium channels, its participation in regulating channel status has not been ruled out. For the remaining proteins that contain the LOV domain, the redox-sensing mechanism is still unknown.

One possible function for the LOV domain in this diverse group of proteins is as a flavin-binding site with the bound flavin acting as a redox sensor. Although NIFL is a flavoprotein (*19*) and Aer is a putative flavoprotein (*20*), we do not yet know whether the FAD attachment site is located within the LOV domain. If the LOV domain is a flavin attachment site, it shares little or no sequence similarity with known flavin-binding sites, such as those of the CRY1/CRY2 blue light photoreceptors (*23*). The LOV domain may also mediate protein-protein interactions, because it shares similarity with PAS domains, which participate in protein-protein interactions (*24*). Regardless of its function, the LOV domain has been conserved during evolution over a wide range of taxa from archaea through higher plants and animals.

Elucidation of the properties and functions of the LOV domain, including the role of two such domains in NPH1, will help unravel the details of the signal-transduction pathway for phototropism.

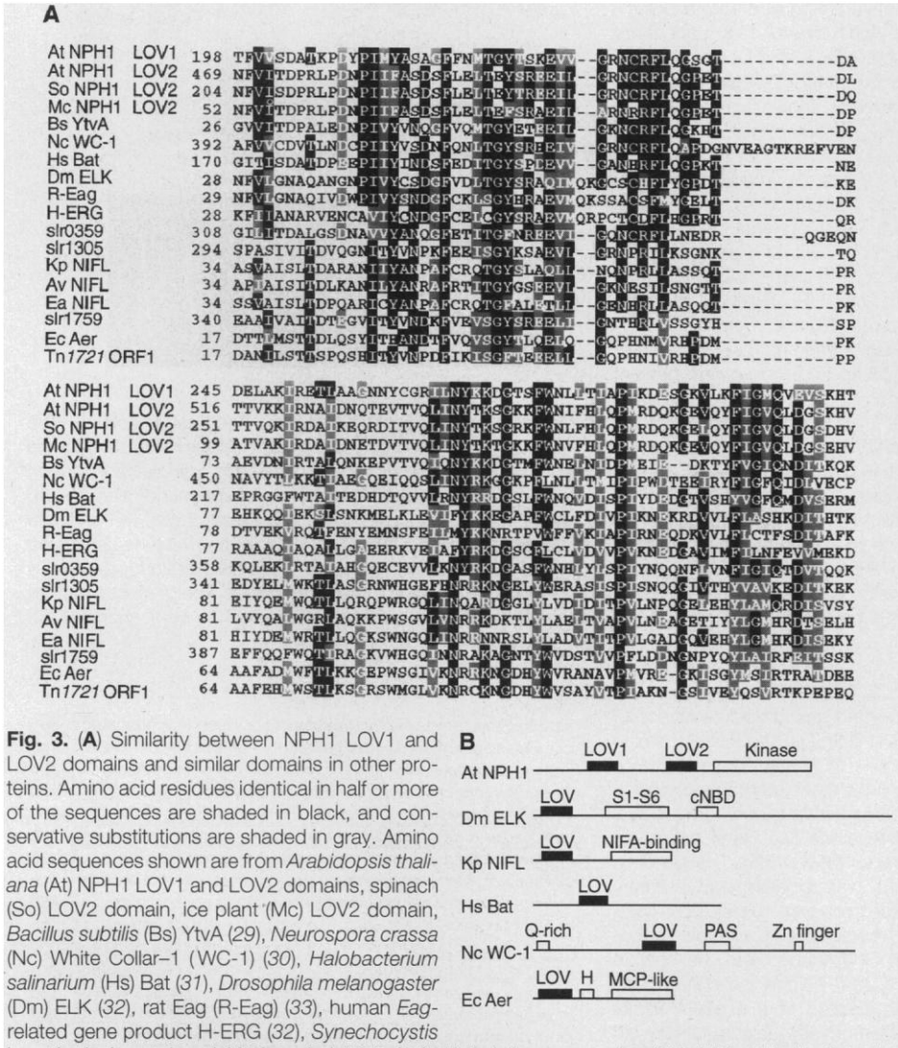


Fig. 3. (**A**) Similarity between NPH1 LOV1 and LOV2 domains and similar domains in other proteins. Amino acid residues identical in half or more of the sequences are shaded in black, and conservative substitutions are shaded in gray. Amino acid sequences shown are from *Arabidopsis thaliana* (At) NPH1 LOV1 and LOV2 domains, spinach (So) LOV2 domain, ice plant (Mc) LOV2 domain, *Bacillus subtilis* (Bs) YtvA (*29*), *Neurospora crassa* (Nc) White Collar–1 (WC-1) (*30*), *Halobacterium salinarium* (Hs) Bat (*31*), *Drosophila melanogaster* (Dm) ELK (*32*), rat Eag (R-Eag) (*33*), human *Eag*-related gene product H-ERG (*32*), *Synechocystis* hypothetical proteins slr0359 and slr1305 (*34*), *Klebsiella pneumoniae* (Kp) NIFL (*35*), *Azotobacter vinelandii* (Av) NIFL (*36*), *Enterobacter agglomerans* (Ea) NIFL (*37*), *Synechocystis* hypothetical protein slr1759 (*34*), *Escherichia coli* (Ec) Aer (*20*), and transposon Tn*1721* open reading frame 1 (ORF1) hypothetical protein (*38*). The alignment was done with the program PIMA 1.4 (*39*). The percentage of similarity to NPH1 LOV1 ranges from 56 for YtvA to 34 for Tn*1721* ORF1. (**B**) Structural features of proteins with LOV domains. Shown are *Arabidopsis* NPH1; *D. melanogaster* ELK, a voltage-sensitive potassium-channel subunit (*32*); *K. pneumoniae* NIFL, a flavoprotein regulator of nitrogenase transcription in response to oxygen (*19*); *H. salinarium* Bat, a regulator of bacterio-opsin in response to oxygen or light (or both) (*40*); *N. crassa* WC-1, a regulator of blue light responses (*30*); and *E. coli* Aer, a putative flavoprotein required for aerotaxis signaling (*20*). LOV domains are shown as black boxes, and other features are shown as white boxes, with abbreviations as follows: kinase, serine-threonine protein kinase domain; S1-S6, six membrane-spanning regions (*32*); cNBD, cyclic nucleotide-binding domain (*32*); NIFA-binding, region that interacts with NIFA (*41*); Q-rich, glutamine-rich region (*30*); PAS, PAS domain (*42*); Zn finger, zinc finger (*30*); H, hydrophobic segment (*20*); MCP-like, resembling the signaling domain of methyl-accepting chemotaxis proteins (*20*).

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Alignment of Conduits for the Nascent Polypeptide Chain in the Ribosome-Sec61 Complex

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An oligomer of the Sec61 trimeric complex is thought to form the protein-conducting channel for protein transport across the endoplasmic reticulum. A purified yeast Sec61 complex bound to monomeric yeast ribosomes as an oligomer in a saturable fashion. Cryo–electron microscopy of the ribosome-Sec61 complex and a three-dimensional reconstruction showed that the Sec61 oligomer is attached to the large ribosomal subunit by a single connection. Moreover, a funnel-shaped pore in the Sec61 oligomer aligned with the exit of a tunnel traversing the large ribosomal subunit, strongly suggesting that both structures function together in the translocation of proteins across the endoplasmic reticulum membrane.

The existence of a protein-conducting channel (PCC) for protein transport across the endoplasmic reticulum (ER) was proposed in 1975 (*1*). Electrophysiological experiments in 1991 provided the first direct evidence for the existence of the PCC (*2*). Moreover, fluorescently labeled nascent chains in membrane-bound ribosomes remain in an aqueous environment sealed from the cytoplasm and accessible to fluorescence quenching from the lumen of the ER (*3*). An aqueous pore with a diameter of 40 to 60 Å during cotranslational translocation is suggested by similar experiments (*4*).

The Sec61 trimeric complex is a strong candidate for the PCC of the ER in yeast and mammalian cells (*5*, *6*). The Sec61 complex provides the principal binding site for ribosomes at the ER during protein translocation (*7*, *8*) and, together with other membrane proteins, is associated with ribosomes after solubilization of rough microsomes with digitonin (*6*). A two-dimensional map of the purified Sec61 complex obtained by electron microscopy has revealed a quasi-pentagonal, circular structure with a central depression (*9*).

The three-dimensional (3D) structure of monomeric ribosomes is currently known at various resolutions for *Escherichia coli* (*10*), wheat germ (*11*), and yeast (*12*). Among the structural features recognized is a tunnel that traverses the large ribosomal subunit and has been considered a candidate for the nascent chain conduit. Here, we present a 3D reconstruction of the ribosome-Sec61 complex.

For purification of the trimeric Sec61 complex (*13*) containing the Sec61α, Sec61β, and Sec61γ subunits (Sec61p, Sbh1p, and Sss1p), a heptameric complex (*14*) was isolated first with protein A–tagged Sec63 protein, followed by elution of the trimeric Sec61 complex with Triton X-100 (Fig. 1A). To determine whether the trimeric Sec61 complex could bind to ribosomes (*15*) in a membrane-free system, we incubated the purified Sec61 complex with ribosomes and analyzed the incubation mixture by sucrose density-gradient centrifugation (*16*). The Sec61 complex incubated without ribosomes remained in the top fraction of the gradient. In the presence of ribosomes, however, the Sec61 complex migrated with ribosomes as determined by immunoblotting (*16*) with antibodies to Sec61β (anti-Sec61β) (Fig. 1B) and Sec61α (anti-Sec61α) (*17*). In agreement with the known salt sensitivity of the Sec61-ribosome interaction, there was no binding at 1 M KOAc (OAc, acetate) (*17*). Incubation of a fixed amount of ribosomes with increasing amounts of Sec61 complex resulted in saturation of ribosome-binding sites (Fig. 1, C and D). On the basis of the amount of Sec61α and ribosomes, we estimate that, at saturation, two to four Sec61 trimers were bound per ribosome and that the dissociation constant K_d is about 10 nM.

The ribosome-Sec61 complex formed under saturating conditions was examined by cryo–electron microscopy (*18*). In the

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