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Drosophila ninaA gene encodes an eye-specific cyclophilin (cyclosporine A binding protein)

(vision/photoreceptor/rhodopsin)

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ABSTRACT **Mutations in the *ninaA* gene of *Drosophila* severely reduce the amount of rhodopsin specifically in R1–6 photoreceptors. Isolation of the *ninaA* gene by chromosomal walking revealed that it is expressed only in the eye and encodes a 237-amino acid polypeptide that shows strong sequence similarity to cyclophilin, a putative molecular target for cyclosporine A, a potent immunosuppressant used in human organ transplantations. Unlike most cyclophilins characterized to date, the *ninaA*-encoded protein has a putative signal sequence and a transmembrane domain. Each of the three ethyl methanesulfonate-induced *ninaA* mutant alleles analyzed shows a single nucleotide change in the mRNA coding region leading to either a nonsense or a missense mutation. We find no evidence that the *ninaA*-encoded protein is directly involved in phototransduction. The only detectable mutant phenotype that correlates with the severity of molecular defects in the three mutants is the amount of depletion of R1–6 rhodopsin. The above results and the recent findings that cyclophilin is a peptidylprolyl *cis-trans*-isomerase suggest that the *ninaA*-encoded protein may be required for proper folding and stability of R1–6 rhodopsin.**

Mutations in a number of genes of *Drosophila melanogaster* have been found to dramatically reduce the amount of visual pigment, rhodopsin (1, 2). In wild-type flies, photoconversion of a substantial net amount of rhodopsin to a thermally stable intermediate, metarhodopsin, results in the generation of a prolonged depolarizing afterpotential (PDA) and concomitant desensitization of photoreceptors (3, 4). The amplitude of the PDA and the amount of desensitization induced are closely, though not linearly, related to the amount of rhodopsin photoconverted (3, 4). Thus, in the mutants with reduced rhodopsin content, the afterpotential and desensitization are underdeveloped or absent (2), because the rhodopsin content is too low for substantial photoconversions to occur. The eight genes identified from such mutations have been named *ninaA* to *-H* (neither inactivation nor afterpotential) (2). One of these genes, *ninaE*, encodes opsin in the R1–6 class of photoreceptors (5, 6), and, therefore, mutations in this gene affect only rhodopsin present in R1–6 photoreceptors. Several lines of evidence, including freeze-fracture electron microscopy and PDA recording from R7 photoreceptors (2, 7), suggest that the effects of mutations in another *nina* gene, *ninaA*, are also confined to R1–6 photoreceptors, suggesting a regulatory function of the *ninaA*-encoded protein on R1–6 rhodopsin. We have undertaken cloning of the *ninaA* gene in the hope of uncovering the identity of its protein product and to learn about its possible role in photoreceptor function.‡

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MATERIALS AND METHODS

DNA and RNA Analyses. General DNA techniques used were as described by others (8). cDNAs were isolated from an adult head library provided by P. Salvaterra (9). Procedures for RNA extraction and Northern blots have been described elsewhere (10).

Chromosomal Walking. A chromosomal walk (11) was initiated by using the clone β-LSP-1 34 obtained from A. Garen (12). Bacteriophages DA4(1), PA1(4), and PA3(2) were derived from the Maniatis library (13). All other bacteriophages were derived from an EMBL 4 library (unpublished data), using an Oregon R stock marked with the white (*w*) (14) mutation. Transcriptional activity within the entire genomic region covered by the walk was tested as follows. ^{32}P-labeled cDNA was synthesized from poly(A)$^+$ RNA from various developmental stages and hybridized to Southern blots containing restriction digests of the region. RNA was extracted from the following developmental stages and adult tissues: embryo (0–24 hr), third instar larvae (5 days), early pupae (5–7 days), late pupae (7–9 days), wild-type adult heads, wild-type adult bodies, and adult heads of the mutant, eyes absent (*eya*) (15).

Chromosomal *in Situ* Hybridizations. Chromosomal squashes were prepared from third instar larval salivary glands as described by Gall and Pardue (16). *In situ* hybridizations were carried out according to the protocol of Langer-Sofer *et al.* (17) with the modifications that DNA was nick-translated using Bio-16-dUTP (Enzo Biochemicals) and hybridization was detected with the Detek-I-HRP detection kit (Enzo Biochemicals).

Construction of Mutant Genomic Libraries. Genomic DNA of mutant flies was isolated as described (10). Purified genomic DNA was digested with *Bam*HI and cloned into *Bam*HI-cut dephosphorylated EMBL 3 λ vector. Genomic fragments corresponding to the wild-type 1.6-kilobase (kb) *Bgl* II fragment were purified and subcloned into M13mp18 for sequencing.

DNA Sequencing and Sequence Analysis. DNA fragments to be sequenced were subcloned into M13mp18 vector, and both strands of all clones were sequenced by the Sequenase system (United States Biochemical). DNA sequence analysis was performed with the programs described by Mount and Conrad (18). DNA and protein data base searches were done with the FASTA program (19) on the BIONET National Computer Resource for Molecular Biology. Hydrophobicity profiles were calculated by the Kyte and Doolittle program

Abbreviations: CsA, cyclosporine A; CyP, cyclophilin; PDA, prolonged depolarizing afterpotential.

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‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. M22851).

(20) available on BIONET. The putative signal sequence cleavage site was determined by using the weight matrix algorithm of von Heijne (21).

Intracellular Recordings. Techniques used for recording intracellularly from *Drosophila* photoreceptors were essentially the same as those described (22).

RESULTS

Genetic Localization and Molecular Cloning of the *ninaA* Gene. Preliminary to cloning, the *ninaA* gene was mapped genetically using the partially overlapping deficiencies *Df(2L)ast-4* (21D1-2;21E1-2) and *Df(2L)ast-6* (21E1-2;21E2-3) (23). Complementation tests showed that *Df(2L)ast-4* does not complement *ninaA* mutations, whereas *Df(2L)ast-6* does (see Fig. 1*A*). The result placed the *ninaA* gene between the distal breakpoints of these two deficiencies (21D1-2;21E1-2) on the left arm of the second chromosome. Because this chromosomal interval contains the previously cloned gene for the β-subunit of the larval serum protein 1 (12, 24), a clone for this gene was obtained and used as a starting point for a chromosomal walk (11). Overlapping genomic fragments were isolated in both proximal and distal directions until the two distal breakpoints were reached (see Fig. 1 *A* and *B*). The positions of the breakpoints were determined by genomic Southern blot hybridizations and *in situ* hybridizations of genomic DNA fragments to giant salivary chromosome squashes of *Df(2L)ast-4* and *Df(2L)ast-6* (data not shown).

Identification and Sequence Analysis of the *ninaA* Gene. Two criteria were used to identify the *ninaA* gene. First, we required that the candidate *ninaA* gene be transcribed specifically in the eye, since the effects of *ninaA* mutations are eye specific; second, we required that, in known *ninaA* mutants, the candidate gene contain nucleotide changes that would lead to alterations of the protein product. To test for eye specificity, we analyzed the transcriptional activity of the entire cloned chromosomal region by hybridizing ^{32}P-labeled cDNA from various developmental sources to Southern blots containing restriction digests of the entire region (data not shown). Only one restriction fragment (1.6-kb *Bgl* II fragment; Fig. 1*C*) was found to be transcribed specifically in adult heads but not transcribed in mutants that lack eyes [eyes absent, *eya* (15)]. Accordingly, this 1.6-kb *Bgl* II fragment was used to screen an adult head cDNA library (9). Sequence analysis of the 1.6-kb genomic fragment and the largest 0.8-kb cDNA isolated (Fig. 1*C*) revealed the presence of a single open reading frame (Fig. 2). The open reading frame, which is interrupted by a 70-base-pair (bp) intron, would encode a polypeptide of 237 amino acids corresponding to a molecular mass of ≈26 kDa. To prove that this is, indeed, the *ninaA* gene, we have isolated and sequenced the same 1.6-kb *Bgl* II fragment from three different ethyl methanesulfonate-induced *ninaA* mutant alleles. Each of the three alleles was found to contain a single base change that would alter the translated product. In the case of *ninaA*^{P263} and *ninaA*^{P228}, nonsense mutations truncate the putative *ninaA*-encoded protein at positions 87 and 208, respectively (Fig. 2). In the case of *ninaA*^{P268}, a missense mutation changes the histidine residue at position 227 into a leucine (Fig. 2). The finding that all three *ninaA* mutations lead to structural alterations of the protein product strongly supports the conclusion that the *ninaA* gene has been cloned.

Transcriptional Analysis. Developmental Northern blot analysis showed that a single 0.95-kb transcript begins to be expressed in late pupae (Fig. 3*A*, lane 4). The expression is eye specific, since the transcript is present in wild-type heads but not in wild-type bodies or *eya* heads (lanes 5–7) and reaches its peak in the adult (lane 6). Comparison of the mRNA expression in the three mutants with that in wild type showed that the putative *ninaA* transcript is not detectable in

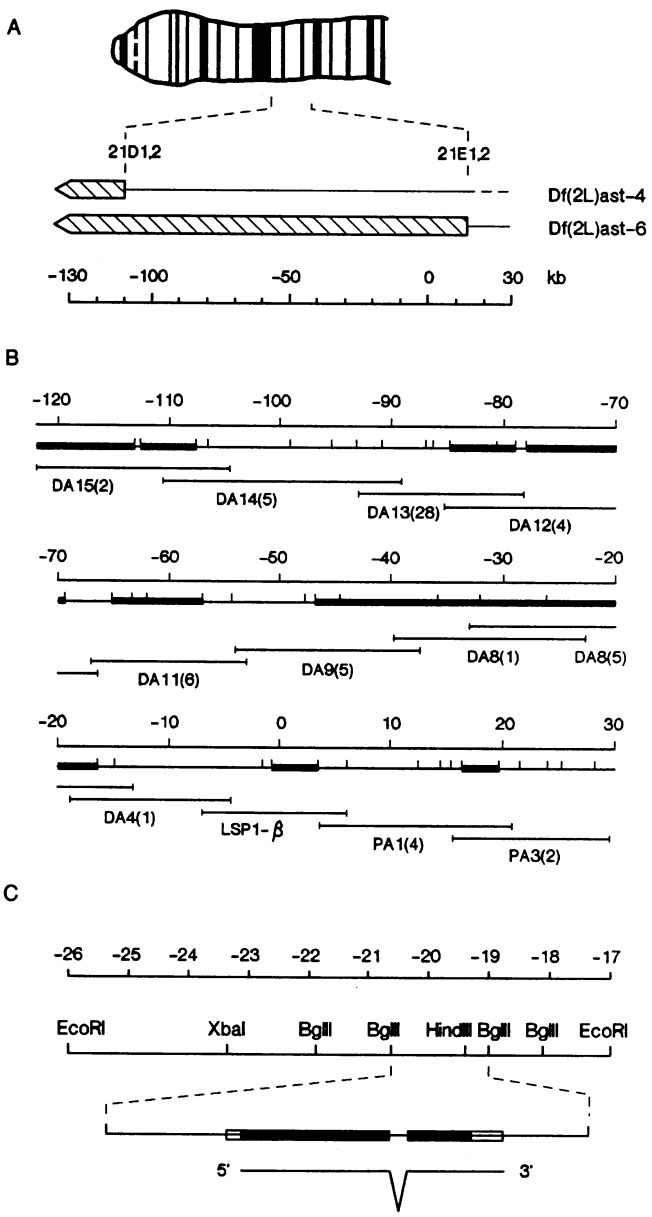

FIG. 1. Genetic localization and molecular cloning of the *ninaA* gene. (*A*) Genetic localization of the *ninaA* gene. Deficiency mapping has localized the *ninaA* gene to the 21D1-2;21E1-2 region on the left arm of the second chromosome between the distal breakpoints of deficiencies *Df(2L)ast-4* and *Df(2L)ast-6* (23). Deficiencies are indicated by thin lines. The proximal breakpoint of *Df(2L)ast-4* has not been cloned, and the uncertainty of its placement is indicated by a dashed line. (*B*) Molecular map of the genomic region 21D1-2 to 21E1-2. The region between the distal breakpoints of *Df(2L)ast-4* and *Df(2L)ast-6* has been cloned by chromosomal walking that spanned 150 kb (see text). The distal breakpoints of *Df(2L)ast-4* and *Df(2L)ast-6* map at positions −110 and +15, respectively. *Eco*RI restriction sites are shown on the map. A detailed restriction map is available upon request. Transcriptionally active fragments are indicated as thick bars. (*C*) Structural map of the *ninaA* gene. The *ninaA* gene is contained within a 1.6-kb *Bgl* II fragment as revealed by cDNA cloning, sequencing, and Northern blot analysis (see Fig. 3). The open reading frame is shown as solid bars and the extent of the full transcript is shown as open bars. The largest (0.8 kb) cDNA isolated is diagrammed below.

ninaA^{P263} but is present in normal amounts in the other two mutants (Fig. 3*B*). The *ninaA*^{P263} mutation is almost certain to be a null allele, because of the undetectable level of *ninaA* mRNA in the mutant (Fig. 3*B*) and the small truncated protein permitted by the nonsense mutation (Fig. 2). The *ninaA*^{P268}

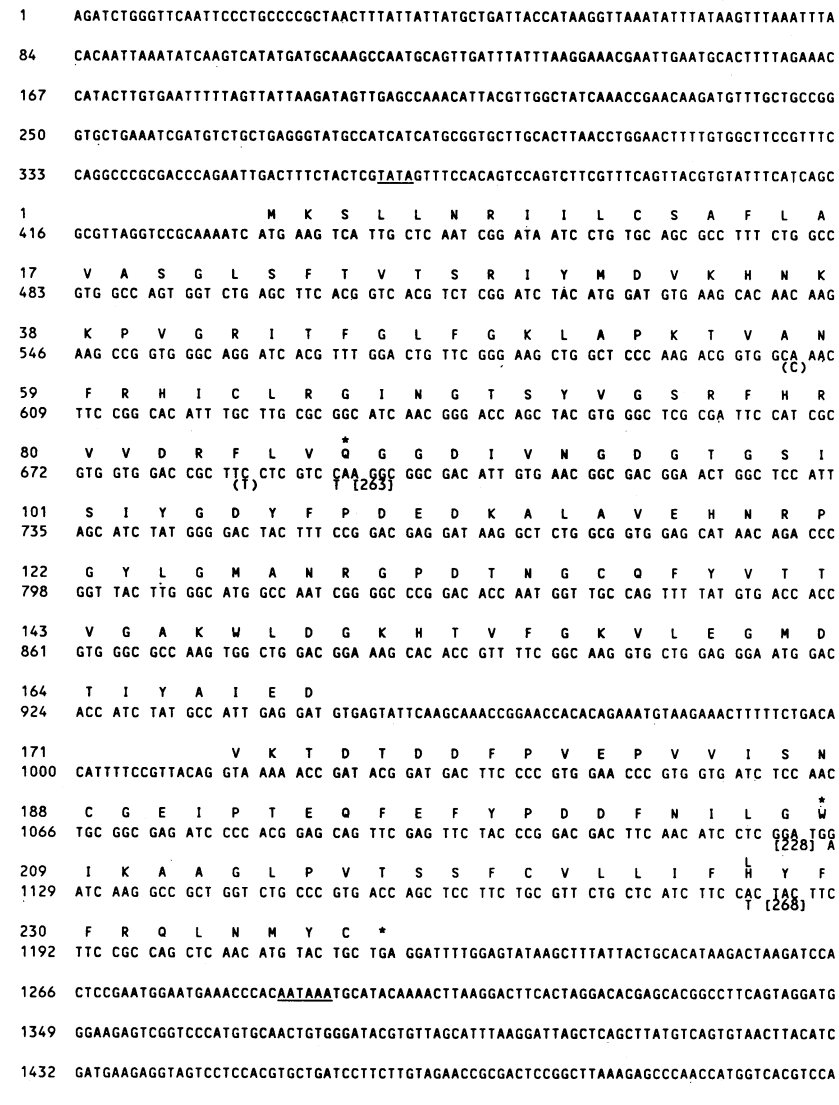

FIG. 2. DNA sequence of the *ninaA* gene. The 1.6-kb *Bgl* II genomic fragment and a 0.8-kb cDNA were sequenced. The gene contains an open reading frame with a coding capacity of 237 amino acids (single-letter code) interrupted by a single 70-bp intron. A possible TATA box and polyadenylylation signal are underlined. Sequence differences between the cDNA and genomic sequences are shown in parentheses. None of the base changes affects the amino acid sequence. The 1.6-kb genomic fragments isolated from genomic libraries of three different ethyl methanesulfonate-induced *ninaA* alleles were also sequenced. Each of the three alleles contains a single base change that results in a change in the primary structure of the protein. The *ninaA*^{P263} allele changes the CAA codon (Q) at position 87 to a TAA stop codon; *ninaA*^{P228} changes TGG (W) at position 208 to a TGA stop codon; and *ninaA*^{P268} changes CAC (H) at position 227 to CTC (L). Stop codons are indicated by asterisks.

allele, on the other hand, is expected to be the least severe of the three alleles since it is the only one that does not cause protein truncation (Fig. 2).

Electrophysiology. To see if there are any electrophysiological defects that correlate with molecular defects determined for the three *ninaA* mutants, the receptor potentials were recorded intracellularly from the R1–6 photoreceptors of the same three molecularly characterized *ninaA* mutants and compared with those obtained from wild type (Fig. 4). It may be seen readily in Fig. 4 that even the null allele *ninaA*^{P263} does not block the generation of the receptor potential. The PDA elicited by the first blue stimulus (arrowheads in Fig. 4), on the other hand, decays much faster in the mutants. In fact, the rapid degradation of the PDA is the only obvious electrophysiological phenotype consistently present in all three *ninaA* mutants. Moreover, the three mutants rank precisely in the same order in terms of the severity of this electrophysiological phenotype as that predicted from the molecular defects. The PDA is generated by a substantial net photoconversion of rhodopsin to metarhodopsin (3), and, therefore, the degree of development of the PDA is closely related to the amount of R1–6 rhodopsin present in the mutants (2), although the relationship is highly nonlinear (2–4). The electrophysiological results thus indicate that the only obvious defects that correlate with the molecular defects in the three mutants are severe depletions of R1–6 rhodopsin. Consistent with this interpretation, microspectrophotometric determinations of the R1–6 rhodopsin content also placed the three mutants in the same order (data not shown). The null allele *ninaA*^{P263}, however, does not eliminate R1–6 rhodopsin completely. There is ≈1% R1–6 rhodopsin still present in the *ninaA*^{P263} mutant.

Sequence Comparison. Comparison of the *ninaA* sequence with those in DNA and protein data bases revealed strong sequence similarity between the *ninaA*-encoded protein and cyclophilin (CyP), a cyclosporine A (CsA) binding protein (Fig. 5). The *ninaA*-encoded protein shares 43% and 45% sequence identity with the human and *Neurospora crassa* CyPs (25, 26), respectively. The larger *ninaA*-encoded protein, however, extends out beyond both the N and C termini of the two CyPs, and the two terminal regions, not present in the two CyPs, contain hydrophobic domains (Fig. 5), which could serve as transmembrane segments (20). The weight matrix algorithm of von Heijne (21) predicts a signal sequence in the N-terminal region of the *ninaA*-encoded protein. The predicted signal sequence cleavage site (Fig. 5) would remove the N-terminal hydrophobic domain, but the C-terminal hydrophobic region could still serve as a membrane anchor for the protein. The sequence similarity notwithstanding, it was felt important to test for the CsA-binding activity of the *ninaA*-encoded protein directly because of the observed topological differences between the *ninaA*-encoded protein and the human CyP. The results of experiments carried out in collaboration with P. Hiestand of Sandoz (Basel, Switzer-

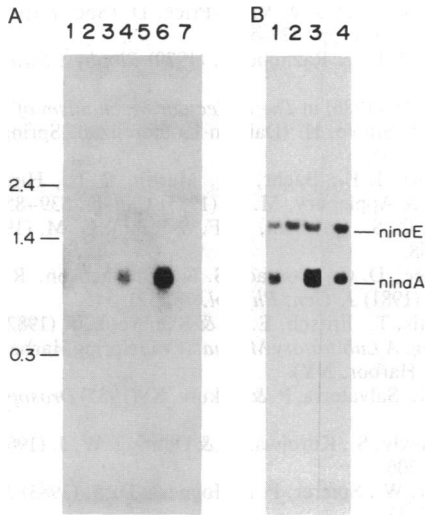

FIG. 3. Northern blot analysis of the *ninaA* gene. (*A*) Developmental profile of *ninaA* mRNA accumulation. Two micrograms of poly(A)$^+$ RNA was loaded into each lane. Lanes: 1, embryo (0–24 hr); 2, third instar larvae (5 days); 3, early pupae (5–7 days); 4, late pupae (7–9 days); 5, wild-type adult bodies; 6, wild-type adult heads; 7, adult heads of *eya* (15) mutant. Absence of hybridization signals seen in some lanes is not due to degradation or poor transfer as tested by rehybridization with various probes. (*B*) *ninaA* mRNA in *ninaA* mutants. Two micrograms of poly(A)$^+$ RNA of adult heads was loaded into each lane. Lanes: 1, *ninaA*^{P268}; 2, *ninaA*^{P263}; 3, *ninaA*^{P228}; 4, wild type. The same filter was rehybridized with *ninaE* (R1–6 opsin) DNA to show that the RNA is intact.

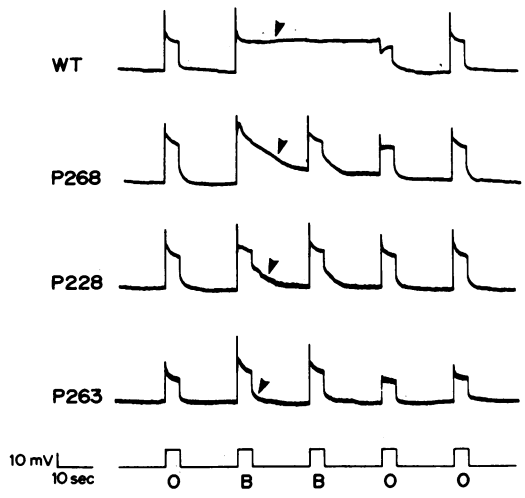

FIG. 4. Electrophysiological phenotypes of wild type and three allelic mutants of *ninaA*. Intracellular recordings of the receptor potentials were obtained from R1–6 photoreceptors of wild type (WT) (first trace), *ninaA*^{P268} (second trace), *ninaA*^{P228} (third trace), and *ninaA*^{P263} (fourth trace). The responses were elicited by a series of orange (O) and blue (B) stimuli of 4-sec durations, as shown in the bottom trace. All flies were marked with the mutation white (*w*) to eliminate the eye color pigments, which otherwise would absorb much of the blue light needed for the generation of the PDA. The PDA generated by the first blue stimulus is indicated by an arrowhead in each trace.

land) showed unequivocally that the *ninaA*-encoded protein, translated *in vitro* in a reticulocyte lysate system, binds specifically to a CsA affinity column (P. Hiestand and S.S., unpublished results).

DISCUSSION

The *ninaA* gene has been found to encode a putative membrane protein with a calculated molecular mass of 26 kDa showing high (45%) sequence similarity to human CyP. CyP has been characterized in mammalian tissues as a ubiquitous cytosolic protein with a molecular mass of 17 kDa (27–29). Because of its high affinity for CsA, CyP is thought to be the primary target for CsA, a cyclic undecapeptide of fungal origin (30) used extensively in human organ transplantation as a potent immunosuppressant (31). Not only is the *ninaA*-encoded protein similar in sequence to human CyP, it also displays high affinity for CsA when translated *in vitro*, suggesting that the *ninaA*-encoded protein, indeed, is a member of the CyP family.

Cellular functions of CyP have not been elucidated, although it has been suggested to play a role in a signaling pathway associated with T-cell activation (32). Thus, it is tempting to suggest that the *ninaA*-encoded protein plays a

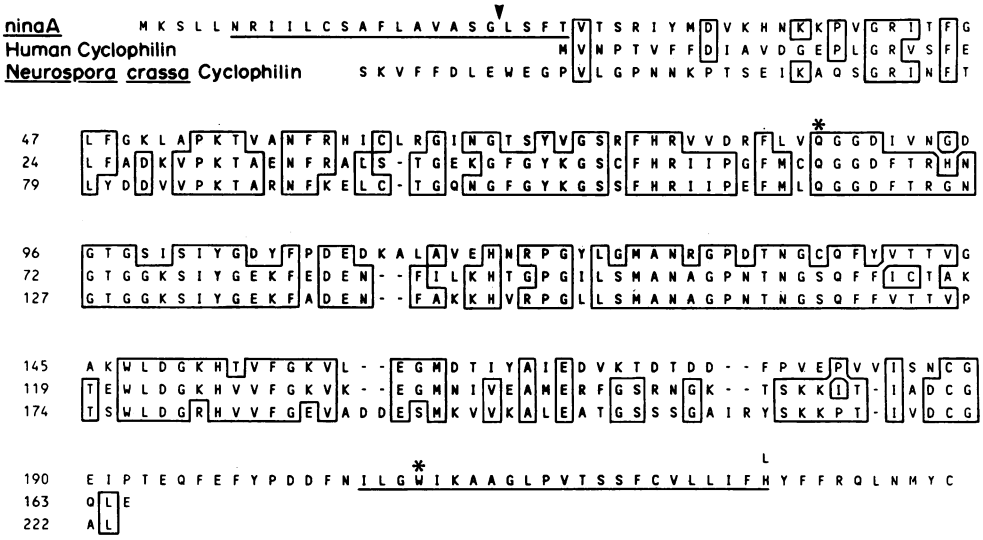

FIG. 5. Amino acid (single-letter code) sequence comparison between the putative *ninaA*-encoded protein and CyP and the effects of *ninaA* mutations on the amino acid sequence. The *ninaA*-encoded protein is aligned with human and *Neurospora crassa* CyP sequences (25, 26). *N. crassa* sequence represents the mature form without the mitochondrial signal sequence. Identical amino acids are enclosed in boxes. The two potential membrane-spanning regions in *ninaA* are underlined, and the putative signal sequence cleavage site is indicated by an arrowhead. The asterisks at positions 87 and 208 mark the sites of protein truncation by stop codons in *ninaA*^{P263} and *ninaA*^{P228}, respectively. The letter L at position 227 indicates the amino acid substitution in *ninaA*^{P268}.

role in phototransduction. If the *ninaA*-encoded protein is directly involved in phototransduction, however, one would expect that a null mutation in the *ninaA* gene would drastically affect the receptor potential. This is clearly not the case (Fig. 5). The slight reduction in the amplitude of the R1–6 receptor potential and the grossly underdeveloped PDA seen in the mutant are both readily explainable in terms of greatly reduced R1–6 rhodopsin content (2, 22). On the other hand, the close correlation between the severity of *ninaA* mutations and the amount of depletion of R1–6 rhodopsin suggests that the *ninaA*-encoded protein may be involved in a process that determines or regulates the amount of rhodopsin in R1–6 photoreceptors. However, *ninaA* mutations have been shown not to affect either the transcription level of the R1–6 opsin gene (*ninaE*) or the *ninaE* mRNA stability (Fig. 4; unpublished results; ref. 33). Thus, the *ninaA*-encoded protein could be involved in the posttranscriptional control of R1–6 rhodopsin levels, such as in the steps required for synthesis or insertion of these molecules into the membrane. It has been established recently that CyP is identical with peptidylprolyl *cis-trans*-isomerase (34, 35), which catalyzes refolding of a number of denatured proteins *in vitro* by proline isomerization. The finding suggests that the *ninaA*-encoded protein may be important for the proper folding and consequent stability of R1–6 rhodopsin.

The *ninaA*-encoded protein is unusual in that it has both a putative signal sequence and a transmembrane domain. Most of the CyPs characterized to date are cytosolic (29) except for one form, which is targeted to the mitochondria through a signal sequence (26). The importance of targeting and site-specific localization of CyPs for their function is further supported by the results on the $ninaA^{P228}$ mutant, in which truncation of the putative membrane anchor alone (Fig. 4) is sufficient to impair the protein function, although the protein is partially functional if the mutant is grown at low temperatures (unpublished observations). It is possible that CyPs discharge their functions through interactions with a target protein and that the target protein for the *ninaA*-encoded protein is R1–6 rhodopsin. Since rhodopsin is a membrane-bound protein, interactions between the *ninaA*-encoded protein and rhodopsin are likely to occur in the membrane, requiring a membrane-bound form of CyP. Further physiological and biochemical analyses of *ninaA* mutants may provide important insights into the role of CyP in cell physiology.

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