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The Cyclophilin Homolog ninaA Is a Tissue-Specific Integral Membrane Protein Required for the Proper Synthesis of a Subset of Drosophila Rhodopsins

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Summary

Mutations in the Drosophila *ninaA* gene cause dramatic reductions in rhodopsin levels, leading to impaired visual function. The ninaA protein is a homolog of peptidyl-prolyl *cis–trans* isomerases. We find that ninaA is unique among this family of proteins in that it is an integral membrane protein, and it is expressed in a cell type–specific manner. We have used transgenic animals misexpressing different rhodopsins in the major class of photoreceptor cells to demonstrate that *ninaA* is required for normal function by two homologous rhodopsins, but not by a less conserved member of the Drosophila rhodopsin gene family. This demonstrates in vivo substrate specificity in a cyclophilin-like molecule. We also show that vertebrate retina contains a ninaA-related protein and that *ninaA* is a member of a gene family in Drosophila. These data offer insights into the in vivo role of this important family of proteins.

Introduction

Cyclosporin A (CsA) and FK-506 are potent immunosuppressants used to prevent graft rejection and to treat autoimmune disorders (reviewed by Shevach, 1985; Thomson, 1989). Although these two molecules are structurally unrelated, they both inhibit similar steps in the activation of T helper lymphocytes (Kronke et al., 1984; Tocci et al., 1989; Trenn et al., 1989; Krensky et al., 1990). Cyclophilin and the FK-506-binding protein (FKBP) are ubiquitous cytosolic proteins with high binding affinity for for CsA and FK-506, respectively (Handschumacher et al., 1984; Siekierka et al., 1989b). Recently, these proteins have generated a great deal of interest as both are peptidyl-prolyl *cis–trans* isomerases (PPIases) (Fischer et al., 1989; Takahashi et al., 1989; Siekierka et al., 1989a; Harding et al., 1989). In vitro, they catalyze the *cis–trans* isomerization about an Xaa-Pro peptide bond (Fischer et al., 1984). This isomerization appears to be the rate limiting step in the in vitro folding of a large number of proteins (Lang et al., 1987; Bächinger, 1987; Davis et al., 1989; Kiefhaber et al., 1990). Interestingly, CsA and FK-506 are potent inhibitors of the PPIase activity of their respective binding proteins (Fischer et al., 1989; Takahashi et al., 1989; Harding et al., 1989; Standaert et al., 1990; Tropschug et al., 1990).

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Although inhibition of the isomerase activity of cyclophilin and FKBP does not appear to be sufficient to prevent lymphocyte activation (Bierer et al., 1990a, 1990b; Sigal et al., 1991), it is widely believed that the immunosuppressive activity of CsA and FK-506 is mediated through their respective binding proteins (reviewed by Sigal et al., 1990).

Insight into the role of cyclophilins in vivo has been gained by analysis of the *ninaA* (for neither inactivation nor afterpotential A) gene product of Drosophila. The *ninaA* gene encodes a 237 amino acid polypeptide that is 42% identical to vertebrate cyclophilin (Shieh et al., 1989; Schneuwly et al., 1989). *ninaA* is essential for proper phototransduction, as mutations in this gene lead to a 10- to 15-fold reduction in the levels of the visual pigment Rh1 rhodopsin (Larrivee et al., 1981). Analysis of *Rh1* gene expression in *ninaA* mutant flies showed that *ninaA* modifies rhodopsin levels posttranscriptionally (Zuker et al., 1988). We now show that ninaA is a membrane protein required for the synthesis of rhodopsin in a subset of photoreceptor cell types. Moreover, we show that vertebrate retina contains a ninaA-related protein and that ninaA belongs to a family of conserved proteins in Drosophila.

Results

The Cloned *ninaA* Gene Rescues the Rhodopsin Deficiency

The Drosophila adult visual system is made up of compound eyes and ocelli. The compound eye is composed of approximately 800 ommatidia, or unit eyes, each containing eight photoreceptor cells: six outer photoreceptor cells, R1–R6, and two central, R7, and R8. The R1–R6 cells express a blue-absorbing rhodopsin, encoded by the *Rh1* gene (Zuker et al., 1985; O'Tousa et al., 1985). R7 cells express UV-sensitive photopigments, either Rh3 or Rh4 (Zuker et al., 1987; Montell et al., 1987), and the ocelli photoreceptors express a violet-absorbing pigment encoded by the *Rh2* gene (Feiler et al., 1988; Pollock and Benzer, 1988). R1–R6 photoreceptors undergo a prolonged depolarizing afterpotential (PDA) that persists after cessation of a light stimulus whenever a substantial amount of rhodopsin is converted to the stable metarhodopsin form (Minke et al., 1975; Hillman et al., 1983). This PDA can be suppressed by photoconverting metarhodopsin back to rhodopsin. During a PDA, photoreceptor cells are refractory to subsequent rhodopsin stimulation and are said to be inactivated. *ninaA* mutant flies have a dramatic reduction of rhodopsin levels in the R1–R6 cells and cannot undergo a PDA (thus the name "neither inactivation nor afterpotential"; Larrivee et al., 1981; Stephenson et al., 1983).

A 2.4 kb EcoRI–BamHI fragment of *ninaA* containing the entire coding region and 1.2 kb of upstream sequence (Shieh et al., 1989) was inserted into the pUChs-neo P element transformation vector (Steller and Pirrota, 1985) and introduced into *ninaA* embryos. Multiple independent germline transformants were obtained and tested for res-

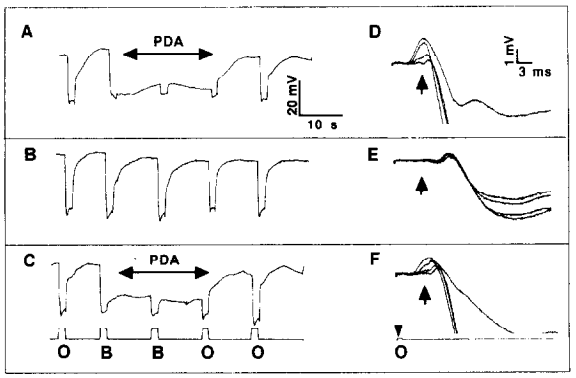

Figure 1. Transformation Rescue of *ninaA* Mutants

Electroretinogram (A–C) and early receptor potential (D–F) recordings of light-evoked potentials to intense orange (O; 580 nm) and blue (B; 480 nm) light were carried out on white-eyed control flies (upper panel), host flies used in the transformation studies (w^{1118};$ninA^{P269}$, middle panel), or host flies transformed with a cloned copy of the wild-type *ninaA* gene (w^{1118};$ninA^{P269}$; P[*ninaA*]12, lower panel). PDA refers to the prolonged depolarized afterpotential. Lower traces indicate the time and duration of light stimuli. Early receptor potential recordings were carried out as described by Minke and Kirschfeld (1979). The position of the M-potential is indicated by an arrow (D–F). The inverted arrowhead indicates the position of the light flash. The superimposed traces represent the amplitude of the M-potential following sequential orange stimuli without intervening reconversion of rhodopsin to metarhodopsin.

cue of the *ninaA* phenotype on the basis of electroretinogram recordings and rhodopsin levels. Figure 1A shows electroretinogram recordings of white-eyed control flies (w^{1118}) displaying a wild-type PDA. Figure 1B shows an electroretinogram of the host w^{1118};*ninaA* mutant flies demonstrating the lack of a PDA and of inactivation. Figure 1C shows a recording of the host flies transformed with a wild-type copy of the *ninaA* gene. The transformed *ninaA* gene fully restores the PDA and inactivation phenotypes. The levels of functional rhodopsin in these transgenic animals were also assayed by recording the early receptor potential (Figures 1D–F); the amplitude of the M-potential in the early receptor potential is an indicator of the functional levels of visual pigment (see Minke and Kirschfield, 1979). *ninaA* mutants transformed with the wild-type gene express nearly wild-type levels of Rh1 rhodopsin in their R1–R6 photoreceptor cells (compare Figures 1D and 1F).

To begin to determine which cell types may require *ninaA* expression, we also generated transgenic flies in which the *ninaA* structural gene was driven by the Rh1 promoter (Mismer and Rubin, 1987), so as to be expressed exclusively in the R1–R6 photoreceptor cells. This construct fully rescued the physiological and morphological abnormalities of *ninaA* mutant flies (data not shown; see Experimental Procedures), demonstrating that expression in R1–R6 cells is sufficient to fully restore Rh1 rhodopsin levels.

ninaA Is Expressed Specifically in Photoreceptor Cells

Extensive genetic, spectrophotometric, and structural data have shown that only the Rh1 rhodopsin levels in the compound eyes are affected by mutations in the *ninaA* locus (Larrivee et al., 1981; Stephenson et al., 1983). The specificity of the *ninaA* phenotype could be due to restricted expression of the *ninaA* gene to the R1–R6 cells and/or to *ninaA* acting only on the Rh1 rhodopsin. To address this question, we examined the sites of expression of the *ninaA* gene using transgenic flies expressing a *lacZ* reporter sequence driven by the *ninaA* promoter.

A gene construct consisting of the *ninaA* promoter and a modified *lacZ* reporter gene including a nuclear localization signal was engineered and introduced into wild-type flies. The inclusion of a nuclear localization signal greatly simplifies cell identification, as the nuclei of the different classes of photoreceptor cells can be easily distinguished by their location in the retina: R1–R6 nuclei are in the distal part of the retina; the R8 nuclei are at the base of the retina; and the R7 nuclei are just proximal to those of the R1–R6 cells. Several independent transgenic animals were obtained and stained for lacZ activity. The *ninaA* gene is expressed exclusively in photoreceptor cells (Figure 2). However, lacZ activity is not restricted to the R1–R6 cells, but is present also in the R7, R8, and ocellar photoreceptors. These results demonstrate that *ninaA* is a tissue-specific cyclophilin and strongly suggest that the specificity of the *ninaA* phenotype cannot be explained by the expression profile of the *ninaA* gene.

The ninaA Protein Is Required by a Distinct Subset of Opsins

The apparent specificity of ninaA for the major class of rhodopsin, Rh1, may be due to substrate specificity for the Rh1 rhodopsin. Therefore, we examined more rigorously how the presence or absence of the ninaA protein affects several classes of rhodopsin. Previous work showed that minor classes of rhodopsin can be ectopically expressed in the major class of photoreceptor cells, R1–R6, by generating transgenic flies containing a transcriptional fusion between the promoter region from the Rh1 rhodopsin gene (*ninaE*) and the structural gene for a minor rhodopsin (Zuker et al., 1988; Feiler et al., 1988). If the *ninaE* gene is deleted in these flies, one can effectively replace the Rh1 rhodopsin with one of the minor opsins; these can then be introduced into either a wild-type or a mutant *ninaA* background. There are several advantages to using ectopically expressed opsins to analyze the effects of *ninaA*. First, when comparing different rhodopsins it is desirable to have them expressed in the same cell type, so they are synthesized and processed in an identical cellular environment. Second, when the expression of minor opsins is directed by the Rh1 promoter region it is expressed at high levels in the major class of photoreceptor cells. Having the rhodopsin "overexpressed" in these cells greatly facilitates the characterization of functional rhodopsin levels (see for example Feiler et al., 1988).

We generated transgenic flies in which each of several different opsins were expressed in the R1–R6 photoreceptor cells. The Rh2 opsin in 70% identical to Rh1 (Cowman et al., 1986) and has an absorption maximum of rhodopsin at 420 nm and of metarhodopsin at 520 nm (Feiler et al., 1988). The Rh3 opsin shares 35% amino acid identity with

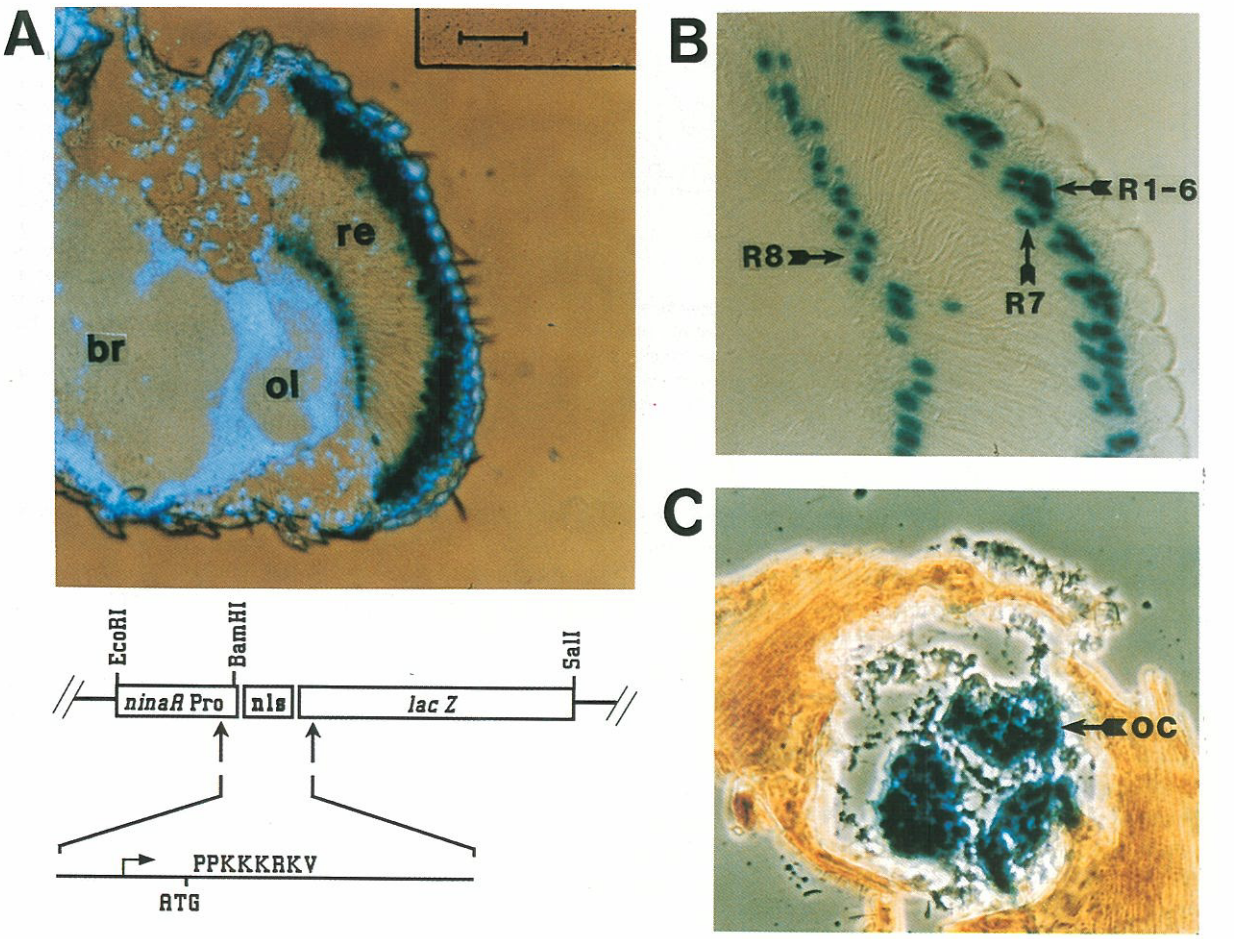

Figure 2. *ninaA* Is Specifically Expressed in Photoreceptor Cells

A DNA construct containing the *ninaA* regulatory region fused to a modified bacterial *lacZ* gene including a nuclear localization signal (nls, see diagram at bottom left) was introduced by P element–mediated germline transformation into wild-type embryos. Shown is the β-galactosidase staining profile of frozen tissue sections. (A) Head tissue counterstained with Hoechst to visualize all nuclei (light blue staining); br, brain; ol, optic lobes; and re, retina. (B) A high magnification view of the retina. R1–R6 refers to the nuclei of R1–R6 cells, and R7 and R8 refer to the nuclei of R7 and R8 photoreceptor cells, respectively. (C) A cross section through the ocelli (oc). Note that staining is present in all photoreceptor cell nuclei. No other sites of expression were detected.

Rh1 (Zuker et al., 1987) and has an absorption maximum of rhodopsin at approximately 355 nm and of metarhodopsin at 450 nm (K. Kirschfeld, R. Feiler, B. Bjornson, and C. S. Z., unpublished data). These rhodopsins are expressed to high levels and are fully functional when targeted to the R1–R6 photoreceptor cells (Figure 3).

Each rhodopsin was expressed in both a strain containing the wild-type *ninaA* gene and a strain homozygous for a null *ninaA* allele. Individual flies were then assayed for the presence or absence of a PDA as an indicator of levels of functional rhodopsin in the different genetic backgrounds. All strains containing the wild-type *ninaA* gene have PDAs (Figure 3, upper traces). However, as one would predict, the wavelength of light required to generate a PDA corresponded to the absorption maxima of each rhodopsin form. As expected for a *ninaA* mutant background, flies expressing the Rh1 rhodopsin in the R1–R6 cells have no PDA (Figure 3, left panel). Interestingly, flies expressing the Rh2 rhodopsin do not have a PDA in a *ninaA* background (Figure 3, middle panel). This demonstrates that production of functional Rh2 rhodopsin, like Rh1 rhodopsin, requires *ninaA*. In contrast, Rh3 rhodopsin is fully independent of *ninaA* activity, as a PDA is observed in both a *ninaA*$^+$ and *ninaA* background (Figure 3, right panel). These results demonstrate that ninaA has in vivo substrate specificity and discriminates among different members of the rhodopsin gene family (see Discussion). Moreover, the normal phenotype of *ninaA* flies expressing Rh3 rhodopsin in the R1–R6 cells demonstrates that all of the general components required for opsin synthesis and phototransduction are functional in these cells.

ninaA Is a Membrane-Bound Cyclophilin

If Rh1 is the substrate of ninaA, they both should be found in the same cellular compartment. Rhodopsins are integral membrane proteins synthesized in membrane-bound ribosomes. The ninaA protein contains highly hydrophobic N-terminal and C-terminal domains, which are not present

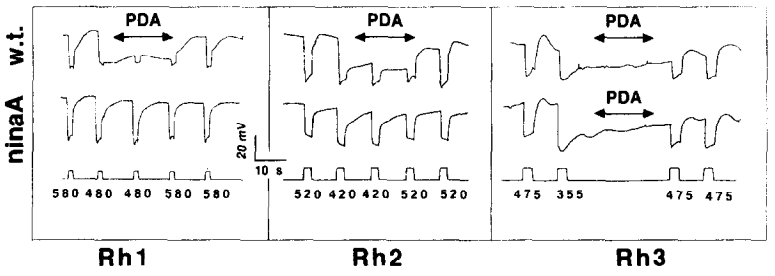

Figure 3. ninaA Is Required by the Rh1 and Rh2 Rhodopsins, but Not Rh3

Shown are ERG recordings from transgenic flies ectopically expressing minor opsins in the R1–R6 photoreceptor cells. The Rh1 promoter was used to direct the expression of Rh1 rhodopsin (left panel) or the structural genes for the minor opsins Rh2 (middle panel) or Rh3 (right panel). The host flies used for P element–mediated transformation were *ninaE*^{117} and thus lacked all endogenous rhodopsin in their R1–R6 cells (O'Tousa et al., 1985). Responses to light stimuli of indicated wavelength (± 15 nm) and of sufficient intensity to trigger a PDA are shown. The absorption maximum of each rhodopsin was obtained by microspectrophotometric analysis (Feiler et al., 1988, and unpublished data). PDAs are indicted by the double arrows. Upper recordings represent wild-type *ninaA* background, and lower traces represent *ninaA*^{P269} mutant background.

The genotypes of the six stocks used are as follows:(top traces) (left) *w*^{1118}, (middle) *w*^{1118}, *P[Rh1+2]#4; nina*^{E117}, (right) *w*^{1118}; *P[Rh1+3]#10, ninaE*^{117}, (bottom traces) (left) *w*^{1118}; *ninaA*^{P269}, (middle) *w*^{1118}, *P[Rh1+2]#4*; *ninaA*^{P269}; *ninaE*^{117}, and (right) *w*^{1118}; *ninaA*^{P269}; *P[Rh1+3]#10, ninaE*^{117}.

in mammalian cyclophilins (Schneuwly et al., 1989). This finding suggests that ninaA might be an integral membrane protein, with the majority of the molecule in the lumen of a membrane compartment (or extracellular) and its C-terminus anchored in the membrane (type 1 as defined by Singer, 1990).

To determine if ninaA is translocated across endoplasmic reticulum membranes, we carried out in vitro translations in the presence or absence of canine microsomal membranes. Figure 4A shows that in the presence of microsomal membranes the ninaA protein increases its apparent molecular weight by approximately 2 kd (compare the first and second lanes), suggesting cotranslational insertion and N-linked glycosylation of the protein (see below). Moreover, this larger form of the protein is fully resistant to protease digestion, demonstrating translocation of the polypeptide chain into the lumen of the microsomes. Because of the increase in its apparent molecular weight in the presence of microsomal membranes, it is unclear whether ninaA contains a cleavable signal sequence. Thus, we constructed a mutant gene lacking the core glycosylation site (Asn^{68}→Gln) and showed that the encoded protein now decreases as opposed to increases its apparent molecular weight in the presence of membranes (Figure 4A, last two lanes). These results demonstrate that ninaA contains a cleavable signal sequence.

The C-terminal tail of ninaA contains a stretch of 20 hydrophobic residues flanked by positively charged amino

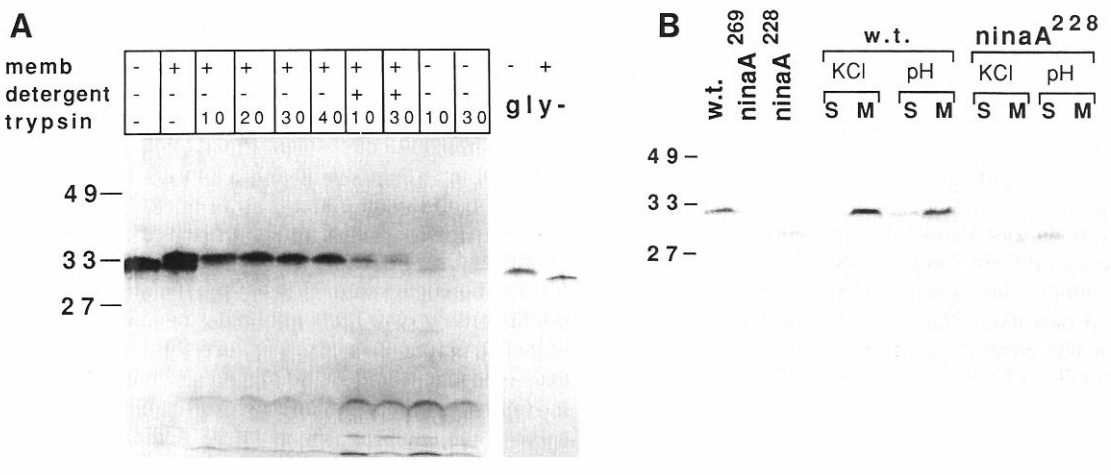

Figure 4. ninaA Is an Integral Membrane Protein

(A) *ninaA* RNA was translated in the presence or absence of canine pancreatic microsomal membranes. Following translation, the products were incubated with 0.1 mg/ml trypsin or trypsin plus 0.1% Triton X-100 to solubilize the membranes and run on a 15% SDS–PAGE. The upper panel indicates the type of treatment and the time in minutes in the presence of trypsin. The glycosylation-defective mutant (gly$^-$) was translated in the presence or absence of microsomal membranes. Numbers to the left refer to molecular weight markers (kd).

(B) Membrane fractions were prepared from eye extracts of wild-type controls or from two *ninaA* mutant strains. *ninaA*^{P228} contains a nonsense termination codon at positiion 207 (Shieh et al., 1989), thus eliminating the C-terminal hydrophobic tail, and *ninaA*^{P269} has a nonsense codon at position 87, generating a null allele. To determine whether ninaA is membrane bound, membrane samples prepared from wild-type and from *ninaA*^{P228} extracts were washed with high salt (1 M KCl) or high pH (sodium carbonate [pH 11.5]), and the soluble (S) and membrane fractions (M) were run on a 15% SDS–PAGE. The gel was transferred to nitrocellulose and probed with a rat anti-ninaA antibody as described in Experimental Procedures. The first three lanes show untreated membrane samples from wild-type and mutant animals; no ninaA protein was detected in soluble fractions of wild-type retinas (data not shown).

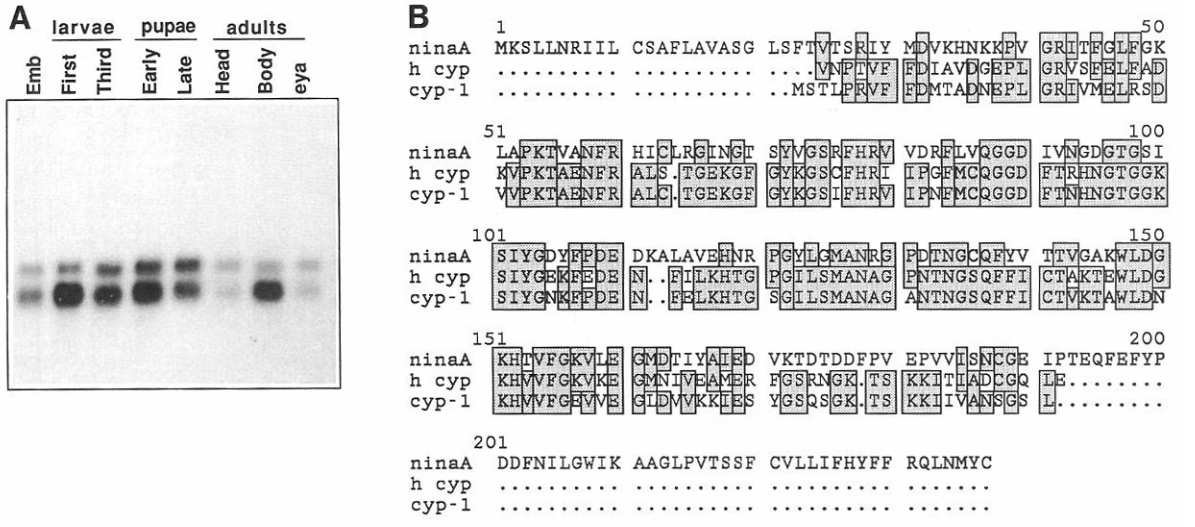

Figure 5. Expression Profile and Amino Acid Sequence of cyp-1

(A) Poly(A)$^+$ RNAs were extracted at different stages of development and from the heads and bodies of wild-type adults. We also isolated RNA from the heads of *eya* flies (Sved, 1986). The RNAs (3 μg per lane) were size fractionated, blotted, and hybridized as described in Experimental Procedures. *cyp-1* hybridizes to 1.0 and 0.7 kb transcripts that are present at all developmental stages. Note that the two RNA species display different tissue distribution (compare heads and bodies). The blot was also hybridized to an actin probe to control for the integrity of the RNA (data not shown). An RNA ladder (BRL) was used as size markers.

(B) Shown is a colinear alignment of the deduced amino acid sequence of the Drosophila *cyp-1* gene, human cyclophilin (h cyp; Haendler et al., 1987), and *ninaA* (Shieh et al., 1989). Amino acids are designated by their single-letter code. The alignment has been optimized for the largest number of identities with the least number of gaps. Boxed areas indicate amino acid identities among the three proteins. Human cyclophilin and cyp-1 display 72% amino acid identity, and ninaA and cyp-1 show 42% identity.

acids, a feature commonly found in integral membrane proteins (Boyd and Beckwith, 1990). To determine whether ninaA is a transmembrane protein, we generated an antibody against a T7 gene 10–ninaA fusion protein (Studier and Moffat, 1986) and examined the localization of the ninaA protein in cellular fractions of wild-type and mutant retinas. Figure 4B shows that the antibody recognizes a 32 kd polypeptide present in wild-type retinal extracts, but missing in extracts from *ninaA* null mutant flies (*ninaA*^{269}). When membranes are separated from cytosolic material by a 100,000 × g centrifugation step, the ninaA protein is found only in the membrane fraction. Moreover, washing these membranes with 1 M potassium chloride or 100 mM sodium carbonate (pH 11.5) (Fujika et al., 1982) fails to release the ninaA protein (Figure 4B). These data, corroborated by the finding that the ninaA protein is extracted by Triton X-114 (M. A. S., unpublished data) (Bordier, 1981), demonstrate that ninaA is indeed an integral membrane protein. We have also examined the localization of ninaA in animals expressing a truncated form of the protein lacking the putative C-terminal membrane anchor domain (*ninaA*^{P228}; Shieh et al., 1989). Interestingly, *ninaA*^{P228} protein is also localized to the membrane fraction (Figure 4B); however, when these membranes are washed with 100 mM sodium carbonate (pH 11.5), so as to break open the microsomes, the protein is now released into the supernatant.

Taken together, these results demonstrate that ninaA is an integral membrane protein with a cleavable signal sequence, and its C-terminus is anchored in the membrane. This structure would generate a protein with the entire cyclophilin homologous domain protruding into the lumen of the ER.

Drosophila also Contain a Homolog of the Vertebrate Cytosolic Cyclophilin

The structure and expression profile of ninaA make it unique among cyclophilins. To determine whether Drosophila also contain a homolog of the ubiquitous cytosolic cyclophilin, we screened a Drosophila cDNA library with the rat cyclophilin sequence (see Experimental Procedures). Figure 5 shows the expression profile and the deduced amino acid sequence of *cyp-1*, a novel Drosophila cyclophilin isolated in this screen. Unlike *ninaA*, *cyp-1* is expressed throughout development and produces two alternatively processed forms (Figure 5A). The absence of a signal sequence and hydrophobic C-terminal domain and the fact that cyp-1 shares 72% amino acid identity with human and rat cyclophilin (Figure 5B) indicate that cyp-1 is a homolog of the ubiquitously expressed mammalian cytosolic cyclophilin.

A nina-Related Protein Is Present in Vertebrate Retinas

The presence of at least two distinct cyclophilin isoforms in Drosophila makes it likely that different organisms have a number of different cyclophilins, each possibly with different substrate and/or tissue specificities. To determine whether vertebrate retina might also contain a ninaA-like protein, we examined bovine tissues for the presence of

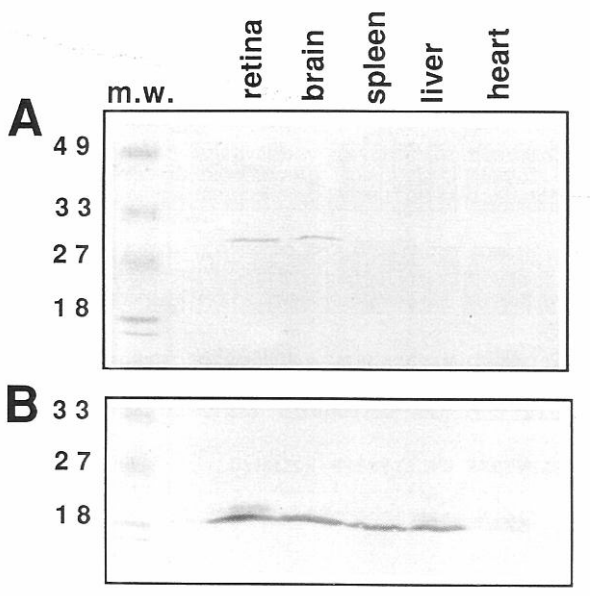

Figure 6. A ninaA-Related Protein Is Present in Bovine Tissues

Membrane fractions were isolated from the indicated bovine tissues and run on a 15% SDS–PAGE (90 μg per lane). Identical immunoblots were then incubated with either (A) rat anti-ninaA antibodies or (B) rabbit anti-rat cyclophilin serum (the rat and bovine proteins are 96% identical). Heart membranes consistently showed low levels of cyclophilin. The brain tissue was cerebral cortex. Molecular weight markers are shown in the first lane.

polypeptides that cross-react with the ninaA antibodies. Indeed, the ninaA antibody recognizes a 30 kd protein present in membrane fractions from bovine retina and cerebral cortex (Figure 6A). This protein is clearly distinct from bovine cyclophilin in its size (30 kd versus 18 kd), abundance, and tissue specificity (see Figure 6B). It is worth noting that the 30 kd protein, which is similar in size to the protein encoded by the *ninaA*^{P228} allele, lacks a membrane anchor domain, as it can be released into a soluble form by alkali treatment of the membranes (M. A. S., unpublished data).

Discussion

The ubiquitous nature of cyclophilin and FKBP suggest that they play a fundamental role in cellular metabolism. The novel enzymatic activity observed for these proteins, namely the ability to catalyze the *cis–trans* isomerization about peptide bonds between proline and its amino neighbor, has led many to speculate that these molecules play a role in protein folding. Although PPIase activity has not been directly demonstrated for ninaA, primarily due to the lack of suitable in vitro substrates, its high degree of homology to cyclophilins makes it likely that it will share this activity. The phenotype of *ninaA* mutant flies, greatly reduced rhodopsin levels, immediately suggests a model for *ninaA* action. Isomerization about an Xaa-Pro bond in rhodopsin may be required for its proper synthesis, folding, transport, or stability.

The results presented in this paper support the model that Rh1 itself is the substrate of ninaA. First, in the compound eyes, *ninaA* is required only in the R1–R6 photoreceptor cells, where Rh1 rhodopsin is synthesized. Second, ninaA is present in the endoplasmic reticulum, where rhodopsin is synthesized. Third, when the ninaA protein is expressed at levels well below wild type (at low temperatures in *hsp70–ninaA* expressing flies), the PDA is restored in transformed mutant flies (E. Baker, M. A. S., and C. S. Z., unpublished data). This suggests that ninaA acts catalytically during rhodopsin synthesis. Fourth, other than Rh1, all other cellular components are functional in the R1–R6 cells of ninaA mutant flies.

The finding that ninaA is not required for the synthesis of all rhodopsins has interesting implications. ninaA may specifically isomerize a peptide bond present on the highly related Rh1 and Rh2 rhodopsins and absent on the more distantly related Rh3 rhodopsin. Several proline residues are conserved between Rh1 and Rh2, but not among all three opsins (Zuker et al., 1987). It is very likely that ninaA activity is also required for Rh2 function in the ocelli. It is also possible that Rh3 and Rh4 rhodopsins require their own specific PPIase. Alternatively, they may not require PPIase for proper synthesis or function. Although substrate specificity has not been observed with other cyclophilins, the FKBP does display significant substrate specificity when assayed with different peptide substrates (Harrison and Stein, 1990).

It is of great interest to determine whether the requirement of ninaA for rhodopsin synthesis is unique to the fly visual system or is a universal phenomenon. The presence of a ninaA-related protein in bovine retinas is consistent with an evolutionarily conserved role for this protein. In this regard, the existence of such a protein may have significant implications in the study of inherited human retinal disorders (for example see Dryja et al., 1990).

A current view of the mode of action of CsA and FK-506 has been that these drugs inhibit the ability of their respective binding protein to catalyze the folding of key components of the signaling cascade involved in T lymphocyte activation. Indeed, the transcriptional activity of a T cell–specific transcription factor, N-FAT, has been shown to be inhibited by CsA and FK-506 (Emmel et al., 1989; Randak et al., 1990; Bierer et al., 1990a). Recently, it has been shown that inhibition of the PPIase activity of mammalian cyclophilin and FKBP is not sufficient for immunosuppression. CsA and FK-506 analogs that bind to their respective binding proteins and inhibit their isomerase activity but do not immunosuppress have been reported (Bierer et al., 1990a, 1990b; Sigal et al., 1990, 1991). These findings suggest that PPIase homologs may have multiple functions: prolyl isomerase activity, required for folding of some proteins such as opsins, and an additional uncharacterized activity such as that required for T cell activation.

The role of immunophilins (immunosuppressant-binding proteins) as the binding proteins for the powerful immunosuppressing drugs CsA and FK-506 has profound medical implications. Moreover, the study of the PPIase activity displayed by these proteins may offer insight into the chemistry of protein folding. The specific phenotype of *ninaA* makes it an ideal model system for the analysis of the cellular role of PPIases. The ability to carry out in vivo

structure–function studies and the possibility of genetically isolating second-site suppressors of *ninaA*, as well as similar studies with *cyp-1*, may provide significant insight into the biology of this protein family.

Experimental Procedures

Fly Stocks

Fly stocks carrying the mutations $ninaA^{P228}$ and $ninaA^{P269}$ were obtained from W. Pak. The original stock $ninaE^{117}$ was obtained from J. O'Tousa. Genetic crosses were carried out under standard laboratory conditions using standard balancer stocks (Lindsley and Grell, 1968). *ninaA* mutant flies were screened morphologically by assaying for the presence or absence of the deep pseudopupil (Franceschini and Kirschfeld, 1971).

In Vitro Translations

Full-length cDNAs encoding wild-type or mutant *ninaA* were cloned into the vector pSP64 (Promega) downstream of the SP6 promoter. The glycosylation-defective ($Asn^{68}\rightarrow Gln$) mutant was constructed using a mismatched oligonucleotide as a primer for the polymerase chain reaction. RNA (5 μg), obtained from in vitro transcription with SP6 RNA polymerase, was translated in the presence of [^{35}S]methionine (Amersham Corp.) in a rabbit reticulocyte lysate system (13 μl per reaction; Promega) at 30°C for 60 min. Canine pancreatic microsomes were obtained from Amersham Corp. Translations were terminated by the addition of 100 μl of 20 mM Tris (pH 7.8) and placed on ice. Aliquots (15 μl) were digested with 0.1 mg/ml trypsin at room temperature for varying lengths of time. The digestion reaction was stopped by boiling in SDS loading buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, 150 mM Tris [pH 6.8]). In control reactions, membranes were solubilized in 0.1% Triton X-100 prior to digestion. Samples were run on 15% SDS–PAGE, and [^{35}S]methionine-labeled products were visualized by fluorography.

Preparation of Samples and Western Blotting

Adult compound eyes (300) were hand dissected and homogenized at 0°C in 300 μl of 20 mM Tris (pH 7.8), 0.1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin 0.5 μg/ml pepstatin using a Dounce homogenizer. The extracts were centrifuged at 300 × g for 1 min to remove the chitin. The supernatant was then centrifuged at 100,000 × g for 30 min at 4° C, and the membrane pellets were resuspended in SDS loading buffer. 1 M potassium chloride, or 100 mM sodium carbonate (pH 11.5). Samples were then incubated on ice for 15 min and recentrifuged at 100,000 × g as before. The supernatant was precipitated with 10% trichloroacetic acid and washed once with ice-cold acetone. All fractions were resuspended in SDS loading buffer and boiled, and the equivalent of 60 retinas was loaded per lane on a 15% SDS–PAGE. The proteins were electroblotted onto nitrocellulose, and the blot was incubated with affinity-purified rat anti-ninaA antibodies and visualized using alkaline phosphatase–conjugated goat anti-rat IgG (Jackson Immunoresearch). Incubations and affinity purification of the antibody were carried out as described (Harlow and Lane, 1988). The anti-ninaA antibody was generated against a bacteriophage T7 gene 10–ninaA fusion protein (Studier and Moffat, 1986) that included residues 81–120 of *ninaA* (Shieh et al., 1989). The fusion protein was purified by denaturing gel electrophoresis as described by Montell and Rubin (1988) and injected subcutaneously into rats.

Bovine tissues were processed by grinding frozen samples, and membranes were prepared as described above for fly retinas.

P Element–Mediated DNA Transformations

Drosophila transformations were carried out exactly as described by Karess and Rubin (1984). Helper DNA was used at a concentration of 200 μg/ml and sample DNA at 1 mg/ml. When using the pUChs-neo vector, stocks were selected and maintained as described by Steller and Pirrota (1985). Constructs in which the *ninaA* structural gene was driven by the Rh1 promoter (Mismer and Rubin, 1987) or the *hsp70* promoter (Lis et al., 1983) used untranslated leaders derived from the exogenous promoters and the entire *ninaA* coding sequence (Shieh et al., 1989).

RNA Blots, Tissue Sections, and Staining

RNA was extracted from embryos, larvae, pupae, or adult flies as described by O'Hare et al. (1983). Heads of wild-type or *eya* adult flies were separated from bodies as described by Oliver and Phillips (1970). Poly(A)$^+$ RNA was isolated, fractionated on formaldehyde gels, and transferred onto nitrocellulose paper exactly as described by Zuker et al. (1988). Hybridizations with labeled DNA probes were carried out at 65°C in 750 mM NaCl, 100 mM NaH_2PO_4 (pH 6.8), 75 mM sodium citrate, 0.04% bovine serum albumin, 0.04% polyvinylpryrrolidone-40, 0.04% Ficoll, 0.5% SDS. Filters were washed in 0.2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 0.5% SDS at 65° × C.

Tissues were sectioned as described by Montell and Rubin (1989). Frozen sections were obtained using a Reichert–Jung 2800 Frigocut-E cryostat (Cambridge Instrument). LacZ staining of transgenic flies expressing the bacterial β-galactosidase gene was carried out as described by Mlodzik et al. (1990).

Electroretinograph Recordings

All recordings were carried out on white-eyed flies to prevent interference from screening pigments. Glass or wick electrodes were filled with standard saline. Light stimulation was by means of a Xenon light beam (450 W Osram, Oriel Corp., Stratford, CT) passed through a high intensity grating monochromator (Oriel model 77264). Unfiltered light intensity was 1.8×10^{-3} W at sample level. Signals were amplified by means of a WPI Dam 60 preamplifier (WPI, New Haven, CT) and digitized on a 1 MHz A/D board (RC-electronics, Santa Barbara, CA). The wavelengths required to trigger a PDA on transgenic flies expressing either the Rh2 or the Rh3 rhodopsin in the R1–R6 photoreceptors were determined experimentally by microspectrophotometry (Feiler et al., 1988, and unpublished data). Early receptor potential recordings were carried out as described by Minke and Kirschfeld (1979). Light source was a high intensity photographic flash (Metz, Inc.).

Isolation of cyp-1 and DNA Sequencing

A Drosophila retinal cDNA library was screened with a radiolabeled rat cyclophilin cDNA (Danielson et al., 1988) in 7 × SSC, 20% formamide, 0.5% SDS at 42°C. Filters were washed in 2 × SSC, 0.5% SDS at 50°C. Positive clones were isolated and subcloned into pBluescript SK$^-$ (Stratagene Inc.). DNA sequencing was carried out by the dideoxynucleotide termination method (Sanger et al., 1977) using Sequenase (US Biochemicals).

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