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The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex *in vivo* with its protein target rhodopsin

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In *Drosophila*, biogenesis of the major rhodopsin, Rh1, is dependent on the presence of a photoreceptor cell-specific cyclophilin, NinaA. In *ninaA* mutants, Rh1 is retained within the endoplasmic reticulum and rhodopsin levels are reduced >100-fold. Cyclophilins have been shown to be peptidyl-prolyl *cis–trans* isomerases and have been implicated in catalyzing protein folding. We have generated transgenic animals expressing different functional rhodopsins containing a histidine tag. We isolated these molecules from wild-type and *ninaA* mutant retinas, and have demonstrated that *in vivo* NinaA forms a specific stable protein complex with its target Rh1. We also expressed *ninaA* under an inducible promoter and showed that NinaA is required quantitatively for Rh1 biogenesis. These results provide the first evidence for a biologically relevant physical interaction between a cyclophilin and its cellular target, and suggest that the normal cellular role of this class of cyclophilins is to function as chaperones, possibly escorting their protein substrates through the secretory pathway.

Key words: chaperone/cyclophilin/*Drosophila*/NinaA/rhodopsin

Introduction

Cyclophilins (CyPs) are a highly conserved family of proteins recognized for two important properties. First, they have peptidyl-prolyl *cis–trans* isomerase (PPIase) activity and, as such, have been implicated in catalyzing rate-limiting steps in protein folding (reviewed by Fischer and Schmid, 1990). Second, they are the intracellular receptors for the drug cyclosporin A (CsA), and mediate its immunosuppressive effects (reviewed by Schreiber, 1991; Walsh *et al.*, 1992). CyPs have a wide phylogenetic distribution spanning the entire animal and plant kingdoms, from bacteria and fungi to cabbage, humans and flies. CyPs have been found in all tissue types examined, and distinct CyP isoforms are residents of many intracellular compartments, including the endoplasmic reticulum (ER), mitochondria, chloroplasts, nucleus and cytosol (reviewed by Galat, 1993; Kunz and Hall, 1993). The ubiquitous and highly conserved nature of CyPs suggests that they play a fundamental role in cellular metabolism (reviewed by Stamnes and Zuker, 1990; Schreiber and Crabtree, 1992; Stamnes *et al.*, 1992).

Much of the research on the biology of CyPs has relied on the use of CsA as a probe to study their structure and function. Surprisingly, the role played by CyPs in the absence of drug appears to be distinct from their role in immunosuppression by CsA. For instance, it is now clear that the drug–protein complex itself mediates immunosuppression: the CsA–CyP complex binds calcineurin and inhibits its calcium-dependent phosphatase activity (Friedman and Weissman, 1991; Liu *et al.*, 1991; Fruman *et al.*, 1992; Liu *et al.*, 1992; Swanson *et al.*, 1992). This in turn prevents the cytoplasmic to nuclear translocation of a subunit of the T cell-specific transcription factor NF-AT (Flanagan *et al.*, 1991), and results in the inability of T cells to undergo activation (Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992; Jain *et al.*, 1993; McCaffrey *et al.*, 1993).

Much of our understanding of the role of CyPs in immunosuppression has come from the study of a structurally unrelated family of immunophilins known as FK506 binding proteins (FKBPs). CyPs and FKBPs appear to mediate T cell immunosuppression by the same mechanism, but FKBP does so by forming a complex with a different drug, FK506. Like CyPs, FKBPs have been highly conserved throughout evolution.

Given the gain-of-function action of the drug–protein complexes, it is possible that CsA (and FK506) recruits CyP (and FKBP) for novel cellular functions it does not normally fulfil. An understanding of the natural cellular role of CyPs will require a biochemical and genetic dissection of their function in the absence of the immunosuppressive drugs or their derivatives.

CyPs and FKBPs have been found associated with a number of different cellular proteins. However, the biological significance of these associations is not understood. For instance, CyP-A has been found complexed with the human immunodeficiency virus type 1 Gag protein (Luban *et al.*, 1993), and CyP-C has been shown to interact with a protein named CyCAP (Friedman *et al.*, 1993). The function of CyCAP is unknown, but it contains a cysteine-rich domain typical of cell-surface proteins. FKBP-12 has been found complexed with the ryanodine receptor, an integral membrane receptor/ion channel involved in calcium excitability (Timerman *et al.*, 1993). P59 (also called FKBP52, or hsp56), another member of the FKBP family, has been isolated in a complex with the two chaperones, hsp70 and hsp90, and with the inactive glucocorticoid receptor (GR; Ku Tai *et al.*, 1992; Yem *et al.*, 1992; Smith *et al.*, 1993). P59 is also found in a complex with an estrogen receptor-binding cyclophilin (ERBC; Ratajczak *et al.*, 1993). Because P59 can be immunoprecipitated with both the intermediate and heavy chains of dynein, it has been suggested that the inactive

steroid receptor and its associated proteins make up a 'transportosome' to facilitate movement of the complex within the cell (Pratt, 1993). An understanding of the biological significance of these interactions will require an *in vivo* study of the role of these proteins and the physiological effect of their loss or dysfunction on cellular metabolism.

Recent studies suggest that CyPs play a role in the stress response. The *CyP-1* gene of yeast contains a heat-inducible promoter, and yeast *CyP-1* and *CyP-2* mutants have a reduced rate of survival following exposure to high temperatures (Sykes *et al.*, 1993). CyP mRNA levels in maize increase in response to various stresses, including mercuric chloride treatment, heat-shock, wounding, salt stress and low temperature (Marivet *et al.*, 1992). In the rat hippocampus, CyP mRNA levels increase in response to lesion-induced seizures (Yount *et al.*, 1992).

One of the best models available for the study of the functions of CyPs *in vivo* is the *Drosophila* NinaA protein. NinaA is a photoreceptor-specific CyP required for the biogenesis of the visual pigment molecule, rhodopsin (Schneuwly *et al.*, 1989; Shieh *et al.*, 1989; Stamnes *et al.*, 1991). In wild-type *Drosophila* photoreceptor cells, the major visual pigment rhodopsin 1 (Rh1) is synthesized in the ER and transported via the secretory pathway to the rhabdomeres (the microvillar light-transducing organelles) where it functions in phototransduction. Immunocytochemical and biochemical studies have shown that in the absence of NinaA, Rh1 accumulates in the ER and is not transported to the rhabdomeres (Colley *et al.*, 1991). The accumulation of immature opsin leads to a dramatic overproliferation of the ER, often seen in other cell types that accumulate improperly folded proteins in the ER (Colley *et al.*, 1991). Eventually, the improperly processed Rh1 is degraded leading to the decreased rhodopsin levels characteristic of *ninaA* mutants.

Fundamental to our understanding of NinaA function and the role of cyclophilins in general, is the demonstration that these proteins interact with their biologically relevant targets. Previously we have shown that both NinaA and Rh1 colocalize to transport vesicles within photoreceptor cells (Colley *et al.*, 1991). Now we have developed an *in vivo* assay to isolate rhodopsin–NinaA protein complexes and demonstrate that this interaction is not only stable, but biologically relevant. By taking advantage of a large collection of *ninaA* mutants (Ondek *et al.*, 1992) we show that the stability of this interaction is dependent on the last six amino acids of the NinaA protein. Finally, we show that both the maturation rate of Rh1 and the morphological state of the photoreceptor cell are tightly linked to the abundance and efficacy of the NinaA protein.

Results and discussion

NinaA binds to Rh1 rhodopsin in vivo

Previously, we have shown that NinaA and rhodopsin localize to the same intracellular compartments (Colley *et al.*, 1991). This finding suggests that both proteins may physically interact. To demonstrate this interaction, we generated transgenic animals expressing a modified rhodopsin gene containing six histidine residues at its extreme C-terminus. These histidine residues bind nickel with high affinity and avidity, enabling the efficient isolation of rhodopsin and associated proteins by affinity fractionation using Ni-NTA columns (Crowe and Henco, 1992). A histidine-tagged Rh1 rhodopsin construct, containing the entire coding region and 2.8 kb of upstream sequence, was inserted into a P-element transformation vector and injected into *ninaE* embryos that lack the endogenous Rh1 gene. Multiple independent germline transformants were obtained and tested for rescue of the *ninaE* phenotype on the basis of Western blot analysis, electrophysiological recordings and sensitivity to light (intensity–response function). Figure 1 shows that transgenic flies expressing the histidine-tagged opsin molecules (P[Tx]) express wild-type levels of rhodopsin (compare lane 1 with other lanes) and restore normal visual physiology to the mutant hosts. These results confirm that the modified Rh1 (Rh1-His) is functional and is expressed in sufficient amounts in the correct cellular location in the fly visual system.

Rh1-His protein can be isolated by homogenizing the heads of transgenic flies and running the detergent-solubilized membrane fraction through a Ni-NTA affinity column. Figure 1C shows that untagged rhodopsin from wild-type control flies is found exclusively in the flow-through (unbound) fraction. In contrast, most of the rhodopsin from animals expressing Rh1-His binds specifically to the Ni-NTA affinity resin and can be eluted by washing the column with the histidine analog, imidazole. The NinaA protein does not contain runs of histidine residues and, as expected, is found in the flow-through fraction of extracts from control wild-type flies (Figure 1C, lower panel). However, when extracts from Rh1-His animals are used, a significant proportion of NinaA copurifies with the tagged rhodopsin molecule. In addition, the association of NinaA and rhodopsin is so stable that it cannot be disrupted even after washing with 2.5 M NaCl (Figure 2A). These results indicate that rhodopsin and NinaA are complexed together, and that the complex is stable enough to be maintained during purification. Interestingly, treatment with 1% β-mercapto-ethanol significantly disrupts the association between NinaA and rhodopsin. This may reflect either the presence of intermolecular disulfide bond(s), or a requirement for the correct 'folded' state in one of these two proteins. In this regard, Khorana and co-workers (Karnik *et al.*, 1988; Karnik and Khorana, 1990) have shown that vertebrate rhodopsin forms an intramolecular disulfide bridge between a pair of highly conserved cysteine residues which are critical for its proper folding.

Although Rh1-His and NinaA clearly interact on the Ni-NTA column, there remains a question as to whether this interaction is biologically relevant. Thus, we prepared membrane extracts from a mixture of heads from two different types of fly: those expressing Rh1-His but lacking the NinaA protein (*ninaA* null mutants), and those expressing normal *ninaA* but lacking rhodopsin (rhodopsin null mutants). Figure 2B shows that in such a mixing experiment the NinaA protein fails to associate with the tagged rhodopsin, even though both proteins are present together during the homogenization and fractionation procedures. These results indicate that the association between NinaA and Rh1-His proteins occurred *in vivo*, and that the extraction procedure does not promote but merely preserves their interaction.

The interaction between Rh1 and NinaA is not only

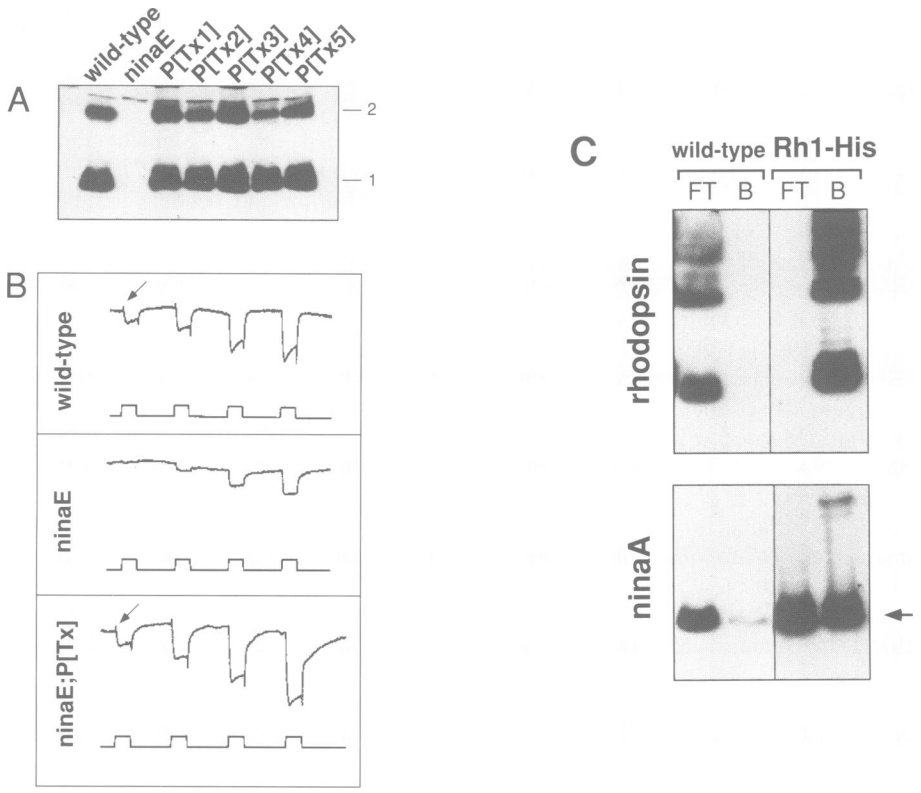

Fig. 1. Rh1-His is functional in transgenic flies. (**A**) Western blot demonstrating normal levels of rhodopsin in flies expressing Rh1-His transgenes. *ninaE* refers to a null allele of the Rh1 structural gene and P[Tx1–5] represent five independent transgenic lines. (1) and (2) refer to the monomer and dimer forms of Rh1, respectively. Note that Rh1-His is expressed at wild-type levels. (**B**) Shown are electroretinogram recordings of responses to log increases in light intensity of 520 nm wavelength (relative intensities are 1/1000, 1/100, 1/10 and 1) for wild-type (top panel), *ninaE* null mutants (middle panel) and *ninaE* nulls expressing the Rh1-His transgene (bottom panel). The small amplitudes seen in *ninaE* mutants at high light intensities represent activity from the R7 and R8 photoreceptor cells. Arrows indicate on-transients resulting from the light activation of the R1–R6 photoreceptor cells (Feiler *et al.*, 1988). (**C**) NinaA complexes with Rh1-His. Protein extracts from wild-type flies or flies expressing Rh1-His were applied to Ni-NTA affinity columns, washed and eluted as described in Materials and methods. 10% of the flow-through (FT) and all of the bound (B) fractions were subjected to PAGE. Shown are Western blots probed for Rh1 (upper blot) and NinaA protein (lower blot). Untagged Rh1 from wild-type flies does not bind to the column and is recovered in the flow-through (FT). In contrast, Rh1-His binds to the column and is specifically eluted with 150 mM imidazole. Silver staining of the bound fraction reveals additional proteins bound to the column (data not shown). This is likely to reflect the binding of cellular proteins with histidine-rich regions, as the same profile is seen in control and Rh1-His extracts. (B) Reprobing the same blot for NinaA (lower panel) shows that NinaA does not bind to the column when extracts containing untagged Rh1 are applied (bottom left panel). However, a significant amount of NinaA protein co-elutes with Rh1-His. These experiments contained extracts isolated from 300 heads.

biologically relevant, but also very specific. 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 of the Rh1 rhodopsin gene and the structural gene for a minor rhodopsin (Feiler *et al.*, 1988, 1992; Zuker *et al.*, 1988). If the *ninaE* gene is deleted in these flies, Rh1 rhodopsin is effectively replaced by one of the minor opsins. Previously we showed that NinaA is required for the synthesis of the two closely related *Drosophila* opsins Rh1 and Rh2, but is not required by the two more distantly related R7 cell-specific Rh3 and Rh4 opsins (Stamnes *et al.*, 1991). To demonstrate that the interaction between NinaA and Rh1 is both biologically relevant and highly specific, we generated transgenic flies expressing histidine-tagged Rh3 in the R1–R6 cells (the normal cellular site of action of NinaA) and tested its ability to interact with NinaA. Figure 2C shows that little, if any, NinaA copurifies with Rh3-His. These results highlight the exceptional ability of NinaA to discriminate between related members of the same protein family, and illustrate how the genetic and physiological specificity of NinaA for Rh1 (and Rh2), but not Rh3 (and Rh4), can be demonstrated at the biochemical level.

The formation of Rh1 and NinaA complexes correlates with the state of NinaA

To gain more insight into the basis of the interaction between NinaA and Rh1, we have taken advantage of a large collection of *ninaA* alleles isolated in a previous saturation mutagenesis screen (Ondek *et al.*, 1992). Our strategy was to mate flies carrying a selected *ninaA* allele to transgenic flies expressing Rh1-His but carrying a null mutation in the *ninaA* gene. Progeny from such a cross contain the *ninaA* mutant allele and Rh1-His in the same photoreceptor cell.

NinaA is an integral membrane glycoprotein containing a cleavable N-terminal signal sequence, a central CyP-homologous domain protruding into the lumen of the ER, a hydrophobic transmembrane domain and an approximately seven amino acid C-terminal tail extending into the cytoplasm (Stamnes *et al.*, 1991; see diagram in Figure 4). From among the mutants isolated in our *ninaA* screen, we recovered two alleles that contained nonsense termina-

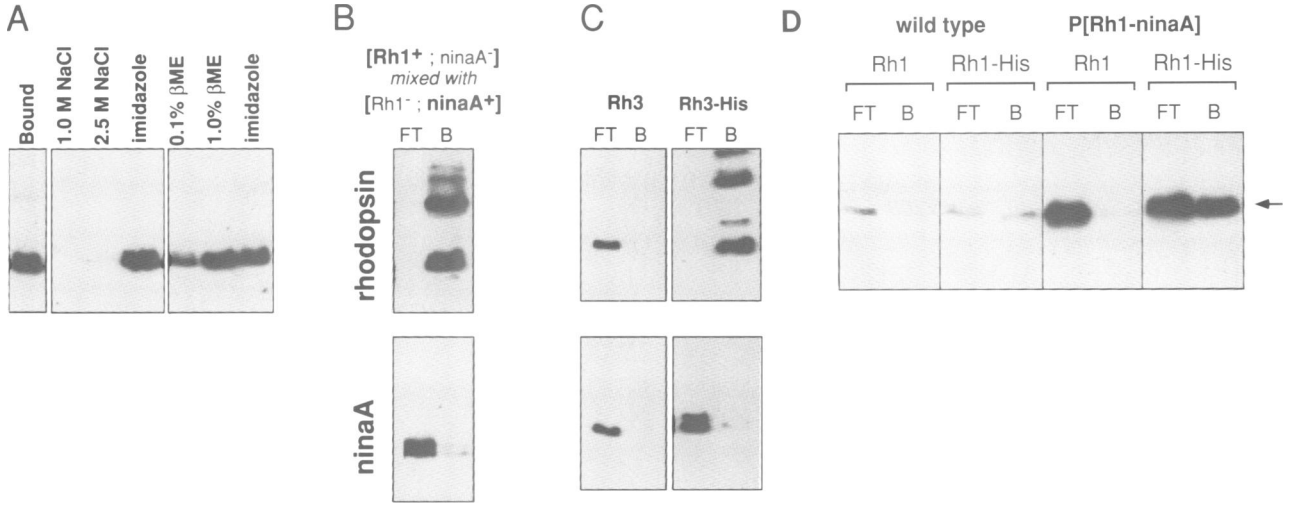

Fig. 2. The NinaA–Rh1 interaction is highly specific. (**A**) Rh1-His–NinaA complexes were bound to Ni-NTA affinity columns as described in Figure 1; the columns were then washed with either increasing amounts of NaCl or β-mercaptoethanol (βME). Shown is a Western blot of these fractions probed for NinaA. Washing the column with high salt concentrations does not remove NinaA from the column, and therefore does not disrupt the Rh1–NinaA complex. However, βME removes a significant amount of NinaA protein from the column, but does not remove bound Rh1 protein (data not shown). Each panel contains extracts prepared from 350 heads. (**B**) 300 heads from flies expressing Rh1-His but lacking NinaA were mixed with 300 heads of flies expressing wild-type NinaA but lacking Rh1 rhodopsin. Samples were homogenized together and prepared according to Materials and methods. Although Rh1-His binds to the Ni-NTA column (top panel), all of the NinaA protein is found in the flow-through (FT) fraction, demonstrating that the Rh1–NinaA complex must form *in vivo* prior to the preparation of the extracts. As in Figure 1, the FT lanes contain 10% of the total. (**C**) Extracts were prepared from 300 heads of transgenic flies overexpressing either Rh3 or Rh3-His under the control of the Rh1 promoter. The top panels (probed with an antibody against Rh3 rhodopsin) show that untagged Rh3 does not bind to the column, while Rh3-His protein specifically binds to the column. The bottom panels show that NinaA does not form a complex with Rh3-His, demonstrating its high substrate specificity for Rh1. (**D**) Western blots of NinaA protein from control w^{1118} extracts (first panel), flies expressing Rh1-His (*ninaA*$^{+}$/*ninaA*^{P269}; *ninaE*$^{+}$/P[Rh1-His]) (second panel), and transgenic flies overexpressing NinaA under the control of the Rh1 promoter (P[Rh1-ninaA]) (third panel). Note the large increase in the levels of NinaA in these animals (all blots contain extracts from the same number of flies). The fourth panel represents flies which express one copy of P[Rh1-ninaA] and Rh1-His (*ninaA*$^{+}$; *ninaE*$^{+}$, P[Rh1-ninaA]/Rh1-His). Thus, P[Rh1-ninaA] flies which express ~10 times more NinaA than wild-type flies also recruit 10 times more total NinaA into Rh1-His complexes (compare second and fourth panels).

tion codons after the CyP-homologous domain but prior to the transmembrane domain (residues Q195 and W208). Interestingly, although both mutants expressed the entire CyP-homologous domain, they were nevertheless strong *ninaA* mutations. The *ninaA*^{W208} allele produced detectable levels of a truncated NinaA product that could be easily distinguished from the wild-type protein based on its smaller molecular weight. Figure 3A (middle panel) shows that ninaA^{W208} fails to associate with Rh1-His. To demonstrate that this truncated molecule does not interfere with the function of the normal NinaA protein (i.e. acting as a dominant negative mutation), we generated flies expressing both NinaA^{W208} and wild-type NinaA protein in the same photoreceptor cells. Figure 3A (right panel) shows that while all of the ninaA^{W208} protein is found in the flow-through, normal levels of the wild-type NinaA protein are found associated with Rh1-His.

The *ninaA*^{Q232} (Ondek *et al.*, 1992) allele contains a nonsense codon that deletes only the last six amino acids of NinaA (QLNMYC); these are presumed to reside on the cytoplasmic face of the ER. Surprisingly, NinaA^{Q232} fails to associate with Rh1-His (Figure 3B). *ninaA*^{Q232} is a very strong allele: photoreceptors from flies carrying this mutation have <1% of the wild-type levels of Rh1 rhodopsin (Ondek *et al.*, 1992), and immunogold labeling of cryosections reveals that most of the Rh1 rhodopsin in these mutants is found predominantly in the ER (Figure 3D). It is possible that the C-terminal cytoplasmic tail is needed for NinaA to interact with the targeting machinery in the cell; therefore NinaA proteins lacking this sequence may be mistargeted in the cell. However, Figure 3C shows that the NinaA^{Q232} protein localizes to the same cellular compartments as the wild-type protein: the ER and transport vesicles. Therefore, despite the fact that both proteins are found in the ER, they do not form a stable complex. Taken together, these results demonstrate that the last six amino acid residues of NinaA are essential for the interaction between these two proteins. This region may be involved directly in the interaction of NinaA with the cytoplasmic face of rhodopsin, or in the association with other components required for the formation of stable Rh1–NinaA complexes. Interestingly, *ninaA*^{S219F} and *ninaA*^{H227L} are two mutations in the transmembrane anchor domain (Ondek *et al.*, 1992) which also behave as very strong *ninaA* alleles. However, NinaA^{S219F} and NinaA^{H227L} still interact with Rh1 (Figure 4A). These mutations may define a region of NinaA required for interacting with other cellular components essential for its function.

In addition to the C-terminal mutants, we also examined 12 mis-sense mutations that mapped to the CyP-homologous domain of *ninaA*. Six of these are strong mutations leading to severe reductions in Rh1 rhodopsin levels (G46R, G88D, G89S, N128Y, G135D and G156D), five are weak (G46E, G98S, G98D, V140E and P179L) and one allele, C188Y, is a temperature-sensitive mutation displaying a near wild-type phenotype at the permissive temperature and a strong *ninaA* phenotype at the non-permissive temperature. The results of these studies are summarized in Figure 4A. All six strong *ninaA* alleles fail to interact with Rh1-His, while all of the weak

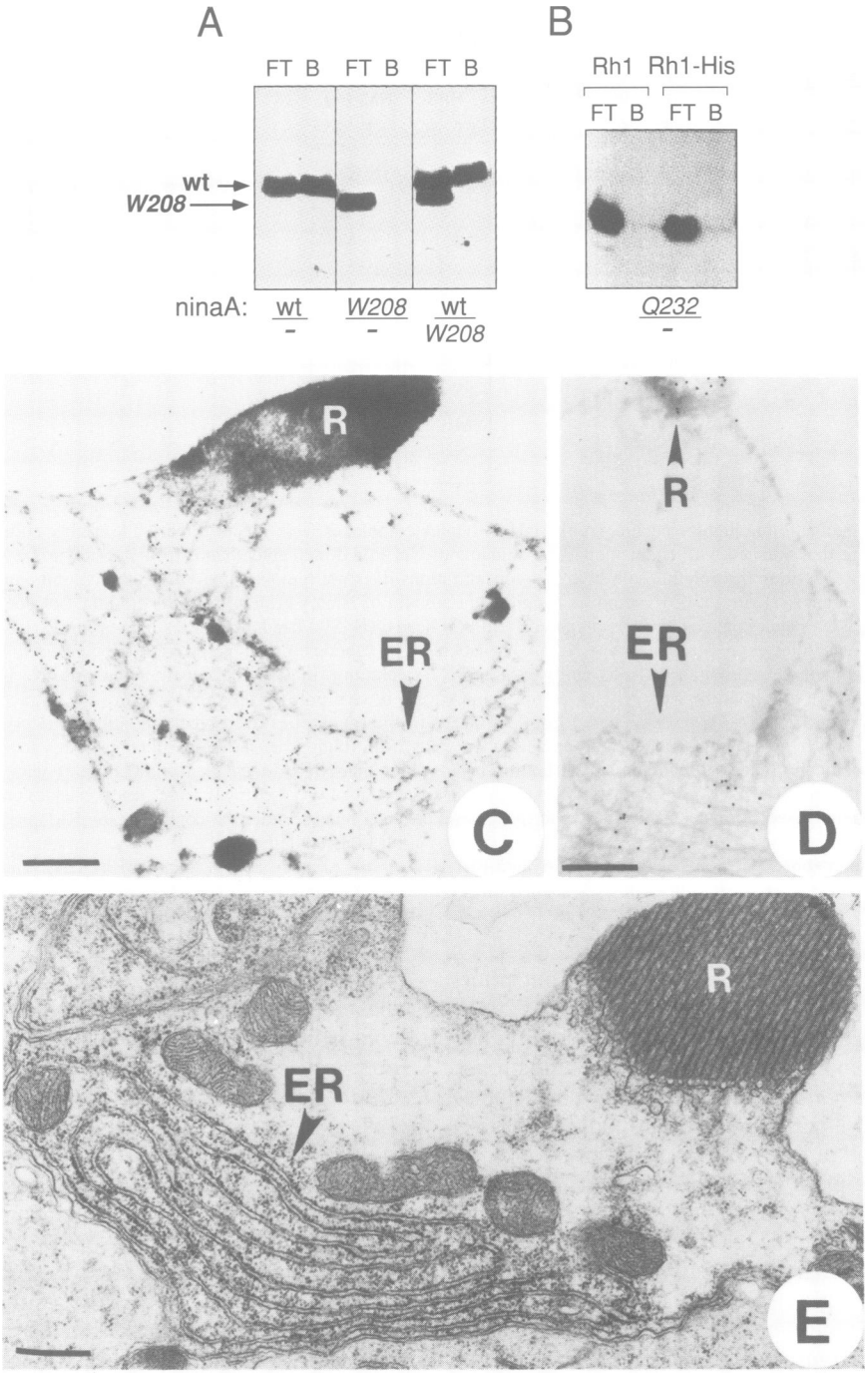

Fig. 3. The C-terminus of NinaA is critical to the Rh1−NinaA interaction. (**A**) *ninaA*^{W208} produces a truncated protein lacking the C-terminal tail that fails to form a stable complex with Rh1-His. Extracts were prepared from 500 heads of the following Rh1-His-expressing stocks. First panel, wild-type NinaA; second panel, *ninaA*^{W208} mutants. Note the smaller size of the protein and its failure to bind Rh1-His. Third panel, heterozygous progeny from a cross between *ninaA*^{W208} and wild-type NinaA which express both the wild-type and truncated forms of NinaA. Even though the wild-type NinaA associates with Rh1-His the truncated NinaA does not, demonstrating the specificity of the NinaA−Rh1 complex. As in Figure 1, the FT lanes contain 10% of the total. (**B**) 1000 heads from *ninaA*^{Q232} mutants were assayed as described in Figure 4B. Like NinaA^{W208}, NinaA^{Q232} fails to bind Rh1-His, indicating that the C-terminus of NinaA is critical for the Rh1−NinaA interaction. (**C**−**D**) Ultrathin cryosections of *ninaA*^{Q232} flies immunolabeled with a rabbit anti-NinaA antibody (C) and a monoclonal anti-Rh1 antibody (D), followed by 5 nm gold-conjugated secondary antibodies. The gold particles were silver-enhanced (Janssen Silver Enhancement kit). (C) NinaA immunoreactivity is in the ER and at the base of the rhabdomeres (bar = 0.4 μm). This indicates that NinaA^{Q232} displays a wild-type distribution within the photoreceptor cell (Colley *et al.*, 1991). (D) A substantial amount of the Rh1 immunoreactivity is in the ER, with some in the rhabdomeres (bar = 0.6 μm). (E) Electron micrograph of a *ninaA*^{Q232} photoreceptor cell displaying large accumulations of ER (bar = 0.3 μm). 'R' refers to rhabdomeres.

alleles form stable complexes with rhodopsin. Furthermore, the temperature-sensitive C188Y allele displays no binding at the non-permissive temperature, but associates with Rh1 at the permissive temperature (data not shown). Together, these results demonstrate a strong correlation between the production of mature functional rhodopsin and the formation of NinaA−rhodopsin protein complexes *in vivo*.

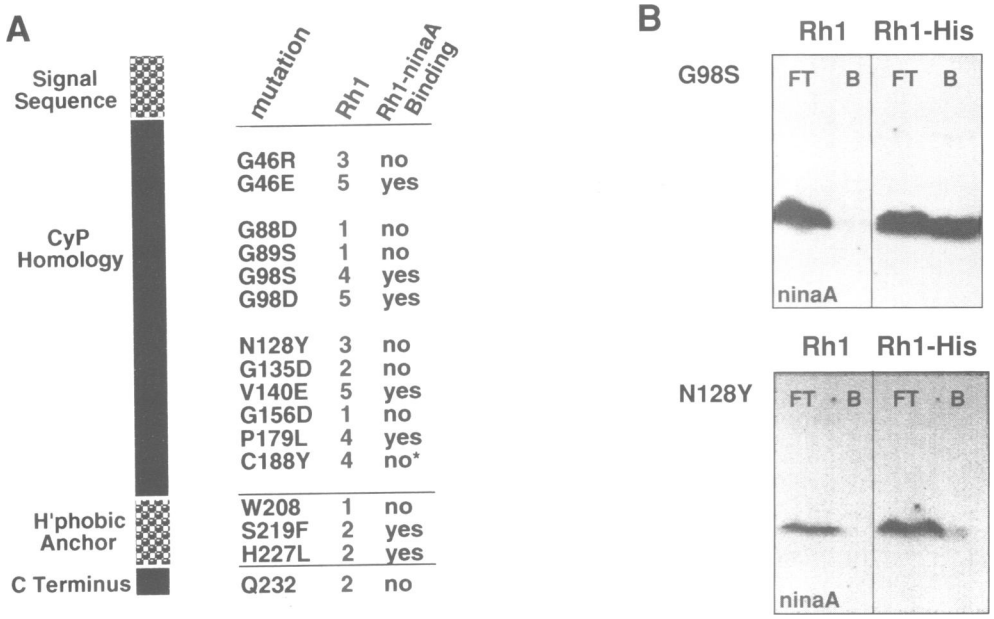

Fig. 4. The interaction of various *ninaA* alleles with Rh1-His. (**A**) NinaA is composed of an N-terminal signal sequence, a CyP 'core' domain, a hydrophobic membrane anchor and a seven amino acid C-terminal cytoplasmic tail. The table shows the sites of several *ninaA* mutations (Ondek *et al.*, 1992) and their levels of expression of Rh1 protein relative to wild-type levels: (1) <7%; (2) 7–25%; (3) 26–50%; (4) 51–75%; (5) >75%. Note that C188Y (*) is a temperature-sensitive allele: the information listed for C188Y refers to flies grown at the non-permissive temperature (29°C). The last column indicates whether each of these mutant NinaA proteins binds to Rh1-His (see text). Most severe *ninaA* alleles do not bind Rh1-His, while weak *ninaA* alleles do. (**B**) For each mutant line listed in (A), flies expressing a mutant form of NinaA and Rh1-His were constructed and extracts were prepared as described in Materials and methods. Each line was then tested for the formation of NinaA–Rh1-His complexes. $ninaA^{G98S}$ is an example of a *ninaA* allele that binds Rh1-His, while $ninaA^{N128Y}$ is an example of a *ninaA* allele that fails to bind Rh1-His. Extracts were prepared from 1000 heads in each case. Rh1 refers to wild-type control rhodopsin.

Rhodopsin:NinaA ratios determine the rate of rhodopsin maturation

The finding that NinaA and rhodopsin form a stable complex suggests that NinaA may function as a chaperone and may be required quantitatively during rhodopsin biogenesis. Previously we have shown that loss of the NinaA protein leads to the proliferation of ER membranes in the cytoplasm of mutant photoreceptors (Figure 5A). This accumulation is due to the presence of improperly processed Rh1 rhodopsin in the ER, as *ninaE* nulls and *ninaA*; *ninaE* double mutants do not display the overproliferation of ER membranes (Colley *et al.*, 1991). The first indication of a quantitative requirement for NinaA in the maturation of rhodopsin came from studies of flies heterozygous for a strong *ninaA* mutation. Photoreceptor cells from such flies, carrying two copies of the wild-type Rh1 gene but only one copy of functional *ninaA*, develop a significant increase in the number of ER cisternae (Figure 5C). To demonstrate that such an accumulation is the result of an imbalance between the normal ratio of rhodopsin:NinaA, we generated doubly heterozygous flies containing one functional copy each of *ninaA* and rhodopsin, so as to re-establish the wild-type ratio. Figure 5B shows that photoreceptor cells from these flies now display normal photoreceptor morphology. These results demonstrate that a 50% reduction in the level of NinaA is sufficient to lead to the accumulation of rhodopsin in the ER, and suggest a non-enzymatic role for NinaA.

If the levels of expression of *ninaA* directly affect the levels of mature rhodopsin, it should be possible to modulate the accumulation of mature rhodopsin by manipulating NinaA levels. Rhodopsin, like most of the other proteins required for phototransduction, is synthesized initially at a high rate during late pupal life and continues into adulthood (Figure 6A; data not shown). Thus, we examined the rate of accumulation of rhodopsin in wild-type animals and in animals expressing different amounts of NinaA.

Drosophila rhodopsin is very stable, with a half-life of >48 h (Schwemer, 1984). Therefore, the levels of mature opsin detected by Western blots are a reliable indicator of its biosynthesis and maturation rates. Figure 6 shows that in wild-type flies most of the rhodopsin is in the mature deglycosylated form (Colley *et al.*, 1991), demonstrating efficient maturation of the visual pigment molecule. Strong *ninaA* alleles (e.g. nonsense mutations that eliminate all gene product $ninaA^{269}$) accumulate only low levels of rhodopsin at all times, and this is almost exclusively found in the high molecular weight endo-H-sensitive immature form (Figure 6B). To determine the effect of varying NinaA levels on rhodopsin biogenesis, we generated transgenic flies containing the *ninaA* structural gene under the control of an inducible heat-shock promoter. NinaA levels were then varied by growing the transgenic animals, either as heterozygotes (one copy of the hs-*ninaA* gene) or homozygotes (two copies of hs-*ninaA*) at 25°C or by inducing high levels of expression using controlled 37°C heat pulses. Figure 6A and C–E shows that the accumulation of mature rhodopsin is indeed dependent on the level of *ninaA* expression. As higher levels of NinaA protein are induced, higher levels of mature rhodopsin are achieved. We also examined Rh1 maturation in a *ninaA* mutant that expresses ~10% of the wild-type levels of NinaA protein ($ninaA^{G46E}$). Interestingly, $ninaA^{G46E}$ is

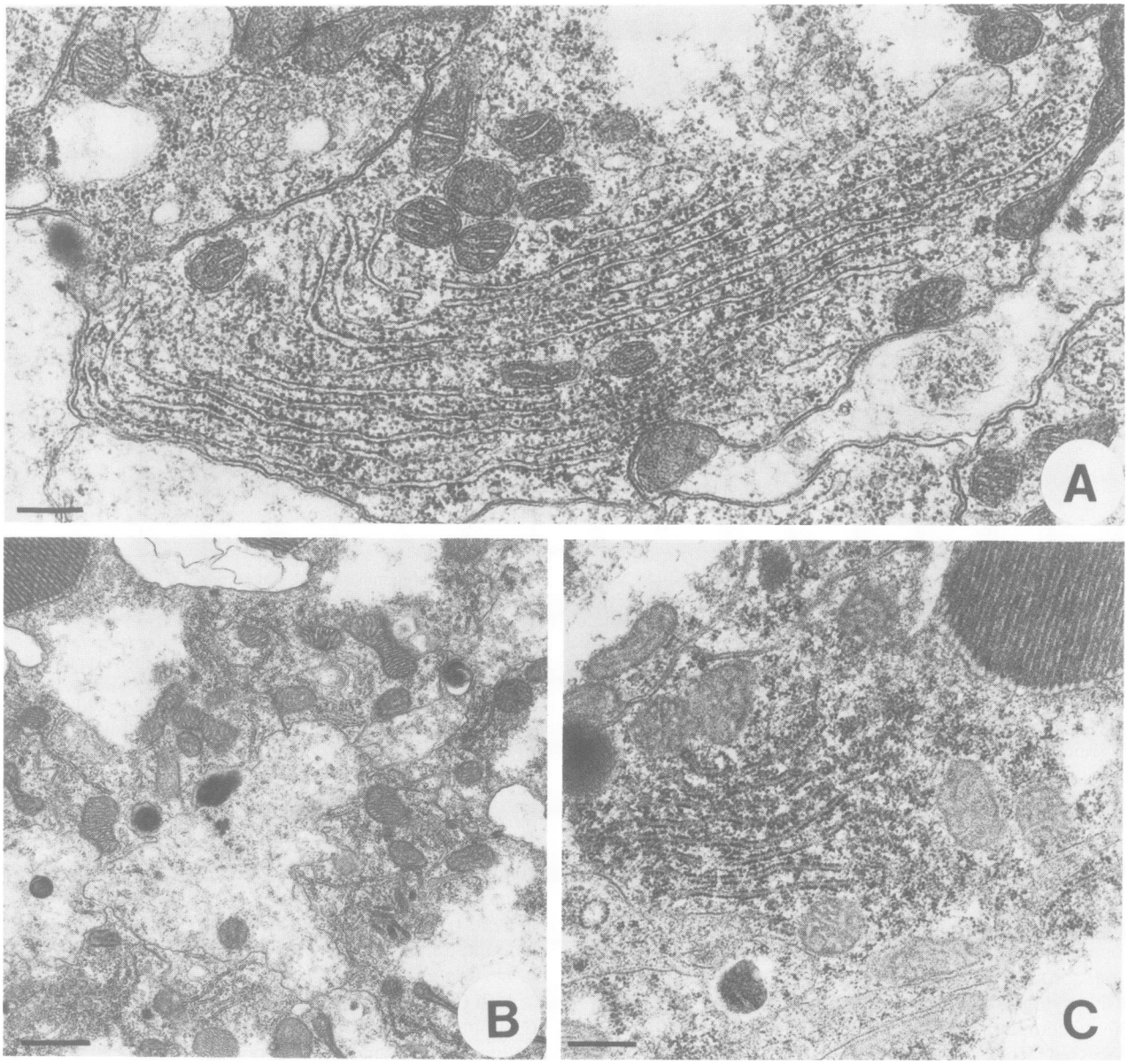

Fig. 5. A reduction in NinaA leads to an accumulation of ER. (**A**) Electron micrograph of a photoreceptor cell from a $ninaA^{P269}$ mutant displaying large accumulations of ER cisternae (bar = 0.25 μm) (see Colley *et al.*, 1991). (**B**) Photoreceptor cells from flies that carry one copy of wild-type rhodopsin and one copy of functional ninaA ($ninaA^{P269}/ninaA^{+}$; $ninaE^{I17}/ninaE^{+}$) display normal ER morphology (bar = 0.5 μm). (**C**) Photoreceptor cells from flies that carry two copies of wild-type rhodopsin but only one copy of functional ninaA ($ninaA^{P269}/ninaA^{+}$; $ninaE^{+}/ninaE^{+}$) display accumulations of ER cisternae (bar = 0.25 μm).

considered a mild ninaA allele since adult mutants contain nearly wild-type levels of Rh1 (Ondek *et al.*, 1992). However, Figure 6F shows that $ninaA^{G46E}$ has severe defects in the rate of accumulation of opsin, clearly evident at the earlier stages of development when the demands for Rh1 biosynthesis are highest. The presence of nearly wild-type levels of Rh1 by 5 days after eclosion is consistent with a model in which NinaA acts 'catalytically' by recycling back and forth within the secretory pathway, and since Rh1 is a very stable protein it would continue to accumulate.

Since rhodopsin maturation is quantitatively dependent on the levels of NinaA, it should be possible to recruit more NinaA into NinaA−rhodopsin complexes by overexpressing NinaA. Indeed, we generated transgenic animals overexpressing *ninaA* in the R1−R6 photoreceptor cells under the control of the strong Rh1 promoter (see Materials and methods) and show that a much larger amount of NinaA now cofractionates with rhodopsin (Figure 2D). Together, these results demonstrate a strict quantitative requirement for the wild-type levels of NinaA during opsin biogenesis. These findings are inconsistent with a solely enzymatic role for NinaA.

Conclusions

The ubiquitous and highly conserved nature of CyPs suggests that they play a fundamental role in cellular metabolism. The demonstration that these proteins display PPIase activity has led to the speculation that they may carry out an enzymatic role in intracellular protein folding. However, despite the wealth of structural information on CyPs, little is known about their roles *in vivo*.

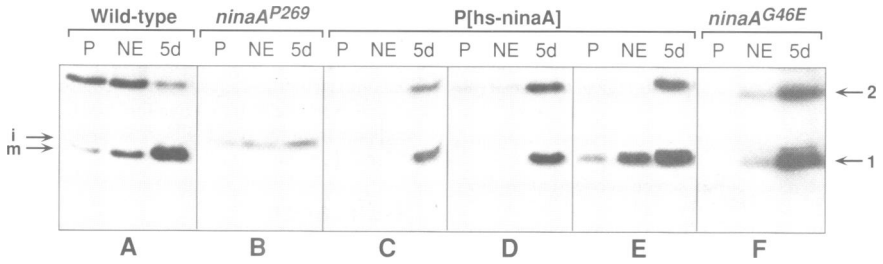

Fig. 6. Wild-type levels of NinaA are critical for the maturation of Rh1. Each lane contains protein extracts from five heads of pupae (P), newly eclosed (NE) or 5 day old (5d) flies (see Materials and methods). (**A**) Rh1 rhodopsin accumulation in wild-type flies. (**B**) *ninaA*^{P269} mutants show a large decrease in the levels of Rh1, with most of the protein found in the high molecular weight immature form (Colley *et al.*, 1991). For clarity, this blot was 5-fold overexposed relative to the other panels. In (**C**–**E**) the expression of the *ninaA* structural gene has been placed under the control of the hsp70 heat-shock promoter (P[hs-ninaA]). Flies expressing one (C) or two copies (D) of the of the P[hs-ninaA] transgene were grown at 25°C which induces low levels of NinaA expression. (E) Flies heterozygous for the P[hs-ninaA] transgene received a 37°C heat pulse for 1 h every 12 h starting during early pupal life. (**F**) *ninaA* ^{G46E}, expressing ~10% of the wild-type levels of NinaA protein, has clear defects in the rate of accumulation of mature Rh1. '1' and '2' refer to Rh1 monomers and dimers, and 'i' and 'm' refer to the immature and mature forms of Rh1 rhodopsin, respectively.

The *Drosophila ninaA* gene offers a unique opportunity for studying the effects of loss of CyP function in a biologically relevant context. This is in contrast to the studies of the gain-of-function effects characterized through the analysis of CsA action on vertebrate CyPs. Genetic, physiological and cell biological analyses have demonstrated an important requirement for NinaA in rhodopsin biogenesis. One possibility is that NinaA may isomerize one or several proline peptide bonds in Rh1 which may be required for its proper synthesis, folding or stability. Alternatively (or additionally), NinaA may act as a chaperone-like molecule escorting rhodopsin through the secretory pathway. In the absence of NinaA, rhodopsin might be improperly folded or translocated and thus not be competent for transport from the ER. Eventually, the improperly processed Rh1 would be degraded in the ER leading to the decreased rhodopsin levels characteristic of *ninaA* mutants. In this paper we sought to determine whether NinaA interacts with its putative target Rh1 rhodopsin, and whether it acts quantitatively.

We have shown that the maturation of Rh1 and the accompanying state of the photoreceptor cell are tightly linked to the quantity and quality of the NinaA protein. We demonstrated that NinaA forms a stable and highly specific complex with rhodopsin. These findings support the model that NinaA acts as a chaperone. The colocalization of NinaA and rhodopsin to transport vesicles (Colley *et al.*, 1991) suggests that NinaA may escort rhodopsin, and be recycled, through the protein trafficking system.

Does NinaA need its PPIase activity for rhodopsin biogenesis? Previous saturation mutagenesis studies of *ninaA* demonstrated that mutations mapping to amino acid residues known to be required for PPIase activity in vertebrate CyP behave as strong *ninaA* alleles (Ondek *et al.*, 1992). Thus, it is possible that NinaA may perform an enzymatic function on Rh1, such as proline isomerization, during transit through the secretory pathway.

Alternatively, NinaA and other CyPs, may utilize their 'PPIase active site' to bind X-Pro residues in their protein targets. If such binding decreases the activation energy for *cis*–*trans* interconversion, the protein would by default behave as an isomerase *in vitro*. However, in such a case binding, but not the PPIase activity, may be required biologically (Schreiber and Crabtree, 1992; Stamnes *et al.*, 1992).

Do other CyPs function as chaperones? Recently, the yeast CYP1 and CYP2 CyPs have been shown to be heat-inducible and to be involved in the heat-shock response (Sykes *et al.*, 1993). The yeast CYP3 is essential for lactate metabolism at high temperature (Davis *et al.*, 1992), suggesting a requirement in the biogenesis of some component of the lactate metabolic pathway. Also, mammalian Cyp-A prevents the aggregation of specific proteins *in vitro* (Freskgard *et al.*, 1992; Lilie *et al.*, 1993). Together, these observations argue that several CyPs may function as chaperones *in vivo*. In this regard, it would not be surprising if mutations in other cell type-specific CyPs reveal defects that parallel the NinaA–rhodopsin interaction.

The requirement for a specific CyP devoted exclusively to rhodopsin biogenesis (even a specific subtype of rhodopsin) can be easily rationalized if one considers that a photoreceptor cell accumulates ~10^8 functional rhodopsin molecules (Johnson and Pak, 1986). Such a massive demand for the efficient expression of a single protein could result in the evolution of a highly specialized optimized machinery for the rapid synthesis and maturation of this molecule.

Materials and methods

DNA constructs and transgenic animals

Rh1-His and Rh3-His contain six histidine residues at the extreme C-terminus of each protein. The modified genes were placed under the control of the Rh1 promoter as described previously (Feiler *et al.*, 1988, 1992; Zuker *et al.*, 1988). Following DNA sequencing of the reconstructed fragments, the modified opsins were subcloned into a P-element transformation vector containing the *rosy*$^+$ gene as a selectable marker (Karess and Rubin, 1984). To generate transcriptional fusions between the *ninaA* structural gene and the Rh1 or heat-shock promoters, PCR mutagenesis was used to introduce *Kpn*I and *Eco*RI restriction sites into the 5′ and 3′ ends of *ninaA*. The modified *ninaA* gene was then ligated to the *Drosophila* hsp70 promoter (Bang and Posakony, 1992) or the Rh1 promoter, and subcloned into a P-element transformation vector containing the neomycin resistance gene as a selectable marker (Steller and Pirotta, 1985).

Drosophila P-element-mediated germline transformation was carried out as described by Karess and Rubin (1984). The Rh1-His and Rh3-His constructs were introduced into flies lacking endogenous Rh1 (*ninaE*^{117}), and selected by the presence of *rosy*$^+$ eye color. The P[hs-

ninaA] and P[Rh1-ninaA] constructs were introduced into flies lacking endogenous NinaA ($ninaA^{P269}$) and selected on food containing neomycin, according to Steller and Pirotta (1985). The expression of these modified genes was confirmed by biochemical, physiological and Western blot analyses.

Fly stocks and transgenic animals

ninaA alleles were obtained in a mutagenesis screen carried out in this laboratory (Ondek *et al.*, 1992). $ninaA^{P269}$ (null allele) and $ninaA^{P228}$ were originally obtained from W.Pak (Larrivee *et al.*, 1981; Stephenson *et al.*, 1983). $ninaE^{117}$ is a null allele of Rh1 (O'Tousa *et al.*, 1985). The wild-type stock used in these studies is w^{1118}. All stocks were constructed using standard balancer stocks (Lindsley and Grell, 1968).

Electron microscopy and immunocytochemistry

Adult heads were fixed and processed according to the method of Baumann and Walz (1989). The fixed tissue was dehydrated in serial changes of ethanol followed by propylene oxide and embedded in Spurr's medium (Polysciences, Inc.). Ultrathin sections were obtained on a Reichert Ultracut E ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate, and viewed at 80 kV on a JOEL 1200EX electron microscope. For all genotypes described, at least five individual heads were sectioned and 100 ommatidia observed from each eye. Immunocytochemistry was carried out as described previously (Colley *et al.*, 1991).

Affinity purification of Rh1-His and Rh3-His

Flies, <5 days old, were frozen in liquid nitrogen and stored at −80°C until needed ($ninaE^{117}$ flies were <12 h old). Heads were isolated as described (Oliver and Phillips, 1970) and homogenized in 20 mM Tris, pH 7.8, 0.01 mg/ml DNase, 0.4 mM PMSF, 0.004 mg/ml leupeptin and 0.004 mg/ml pepstatin in a ground glass homogenizer. Membranes were prepared by centrifugation at 100 000 *g* for 30 min. The pellet was resuspended in 10 ml column buffer (1% *n*-dodecyl-β-D-maltoside, 50 mM sodium phosphate, pH 7.8, 50 mM sodium chloride, 10% glycerol, 0.1 mM PMSF, 0.001 mg/ml leupeptin, 0.001 mg/ml pepstatin) and centrifuged at 25 000 *g* for 10 min to remove insoluble material. Supernatant was loaded onto a 0.15 ml column of Ni-NTA resin (Qiagen). The column was washed with 100 vol (15 ml) of column buffer, followed by 100 vol of buffer plus 10 mM imidazole. Bound protein was eluted with 0.8 ml of column buffer plus 150 mM imidazole. NaCl and β-mercaptoethanol washes (Figure 2A) were carried out in 1 ml of column buffer. All procedures were carried out on ice or at 4°C. A measurement of the stoichiometric relationship between NinaA and rhodopsin has been impossible to obtain due to the fact that it is not feasible to generate tagged functional ninaA molecules (data not shown) and the unavailability of NinaA-immunoprecipitating antibodies. Since only non-rhabdomeric rhodopsin interacts with NinaA, accurate stoichiometry cannot be derived through the purification of rhodopsin.

Protein gels and Western blotting

In all, 10% of the flow-through (FT) and all of the bound (B) fractions were precipitated by adding deoxycholate to 0.02%, followed by trichloroacetic acid to 8%. After a minimum of 30 min on ice, samples were centrifuged at 15 000 *g* for 5 min. The pellets were washed with ethyl ether, air-dried, resuspended in sample buffer (125 mM Tris, pH 6.8, 2% SDS, 8 M urea, bromophenol blue) and sonicated. For Figure 6, fly heads were hand dissected and sonicated in sample buffer. Samples were loaded on a 12% SDS−PAGE (Laemmli, 1970). Western blot transfer was performed according to Towbin *et al.* (1979). Nitrocellulose was placed in blocking buffer (5% milk, PBS, 0.1% Tween) and incubated overnight with a polyclonal antibody against NinaA, Rh1 or Rh3 (Feiler *et al.*, 1992). After washing in PBS/Tween, blots were incubated with HRP-conjugated anti-rat or anti-rabbit antibodies (Jackson ImmunoResearch). Blots were developed using the ECL system (Amersham). To reprobe a blot with a different antibody, the blot was washed in 10% peroxide/PBS for 10 min, washed with PBS, reblocked in blocking buffer and reincubated with a new antibody. The formation of rhodopsin dimers and multimers in PAGE is an artifact of sample preparation (Colley *et al.*, 1991; Stamnes *et al.*, 1991; E.K.Baker and C.S.Zuker, unpublished observations).

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