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Genetic Dissection of Cyclophilin Function

SATURATION MUTAGENESIS OF THE *DROSOPHILA* CYCLOPHILIN HOMOLOG ninaA*

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Cyclophilins, the intracellular receptors for the widely used immunosuppressant cyclosporin A have been found to be peptidyl-prolyl *cis/trans* isomerases and have been implicated in intracellular protein folding and trafficking. The *Drosophila ninaA* gene encodes a photoreceptor-specific cyclophilin homolog involved in rhodopsin biogenesis. ninaA mutants have a 90% reduction in the levels of Rh1 rhodopsin. To gain insight into the role of cyclophilins *in vivo*, we carried out a genetic screen designed to identify functionally important regions in the ninaA protein. Over 700,000 mutagenized flies were screened for a visible *ninaA* phenotype and 70 independent mutations in *ninaA* were isolated and characterized. These mutations provide a detailed dissection of the structure/function relationships in cyclophilin. We also show that mammalian cyclophilins engineered to contain missense mutations found in two temperature-sensitive *ninaA* alleles display temperature-sensitive prolyl *cis/trans* isomerase activity.

Cyclophilins (CyPs)[1] make up a highly conserved family of ubiquitously expressed proteins which are thought to be the cellular targets responsible for the immunosuppressive properties of cyclosporin A (CsA) (reviewed in Refs. 1–3). These proteins share several properties: most display high affinity and stereo-specific binding for CsA (4, 5), and all have peptidyl prolyl *cis/trans* isomerase (PPIase) activity (6, 7). PPIases (also known as rotamases) are enzymes that catalyze the *cis/trans* isomerization of a peptide bond between a proline and its N-terminal neighbor (8). This isomerization has been shown to be the rate-limiting step in the *in vitro* folding of a number of proteins (9–13).The molecular details of the mode of action of CsA in immunosuppression are not yet known. The CyP·CsA complex blocks activation of T-lymphocytes by interfering with intracellular signalling events downstream of T cell receptor activation (see Ref. 2). CsA is a potent inhibitor of the PPIase activity of CyP (6, 7); this inhibition was initially thought to be responsible for immunosuppression. However, CsA analogs that bind to CyP and inhibit its PPIase activity but are not immunosuppressants have been identified (14), demonstrating that inhibition of CyP's isomerase activity is not sufficient to mediate immunosuppression.

A number of recent genetic and biochemical studies have shown that the immunosuppressive action of CsA is mediated by a dominant activity of the CyP·CsA complex, rather than simply by the inhibition of CyP by CsA. For instance, in *Neurospora* and yeast (15) CsA is lethal causing an irreversible block in the cell cycle. CsA-resistant mutants have been isolated in both organisms, and in most cases these survive because of the loss of CsA binding or total loss of CyP (15). These results demonstrate that the lethality is mediated by the CyP·CsA complex rather than the inhibition of CyP function. Friedman and Weissman (16) and Liu *et al.* (17) have used CyP·CsA affinity matrices to isolate cellular proteins that bind to the CyP·CsA complex but not to free CyP. Calcineurin, a calcium-calmodulin-dependent serine/threonine phosphatase, was shown to be a specific target of the CyP·CsA complex. Interestingly, calcineurin's phosphatase activity is inhibited with high specificity by the CyP·CsA complex, suggesting that it may be a biologically relevant target of the CyP·CsA complex (17). In this regard, CsA has been shown to inhibit the cytoplasmic to nuclear translocation of a T-cell-specific transcription factor (NFAT; 18, 19), and protein phosphorylation may be involved in this process.

Dissecting the role of CyPs in cellular physiology will require not only a detailed study of the effects mediated by CsA, but in addition, a detailed analysis of the natural cellular function of CyPs. It has been proposed that CyPs play a role in intracellular protein folding (see for example Refs. 20–22), trafficking (19, 23), and secretion (24). Insights into the *in vivo* role of cyclophilins have come from studies of the *ninaA* gene in *Drosophila* (1, 25). Mutations in the *ninaA* gene result in a major reduction in the levels of Rh1 rhodopsin in the R1–6 photoreceptor cells (26–28). ninaA protein is specific for the Rh1 visual pigment and is required for its export from the ER (23, 29). Previous studies have shown that ninaA is a photoreceptor-specific integral membrane CyP with the cyclophilin-homologous domain located in the lumen of the ER and intracellular transport vesicles (23, 29). Our current view is that ninaA is involved in some aspect of rhodopsin folding. That is, in *ninaA* mutants Rh1 rhodopsin is not competent for transport and as a result immature opsin protein accumulates in the ER and is then rapidly degraded. Although ninaA is unique among CyPs in its high tissue and substrate specificity, it shares high amino acid sequence homology with

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[1] The abbreviations used are: CyP, cyclophilin; PPIase, peptidyl prolyl *cis/trans* isomerase; CsA, cyclosporin A; ER, endoplasmic reticulum; EMS, ethylmethane sulfonate; DPP, deep pseudopupil; SDS, sodium dodecyl sulfate.

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mammalian CyPs (1) making it an ideal model for the study of cyclophilin function *in vivo*.

We now present results of a near saturation genetic screen designed to identify functionally important regions in the ninaA protein. We have isolated and characterized 70 independent mutations in *ninaA*. This screen, based on a rhodopsin-dependent phenotype and unbiased in its requirement for a defined biochemical activity of ninaA, has provided significant insight into the structure/function relationships important to the natural cellular function of a CyP.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetic Screen—*ninaA*^{269} and *ninaA*^{228} mutants were originally obtained from W. Pak (Purdue). The mutagenized stock used in this study was *cn bw*. All crosses were carried out under standard laboratory conditions. Mutagenesis was carried out by feeding *cn bw* males, isogenic for their second chromosome, 25 mM ethylmethane sulfonate (EMS) in 1% sucrose (30). Mutagenized males were mass mated to w^{1118}; *ninaA*^{269} *b* females. Flies were kept 3 days at 25 °C, the females transferred to new bottles and the original cultures transferred to 29 °C. F1 males were scored for a deep pseudopupil (DPP) under blue light illumination (31). Screened males displaying reduced or absent DPP were mated to *SM5, Cy cn* females. F2 Cy *cn* males were then mated to w^{1118}; *Df(2L)S3/CyO* females (32) and their progeny retested under blue light illumination. The mutagenized *cn bw* chromosomes were passed through *w; Df(2L)S3* females to recombine away unlinked lethals. Although all mutants were first isolated over a *ninaA* null allele, the final quantitation of DPP, Rh1 rhodopsin and ninaA levels was carried out in homozygous flies. DPP scorings were performed by four independent investigators in double-blind tests. All weak ninaA alleles were reproducibly identified in independent scoring sessions.

Isolation of Calliphora ninaA, Drosophila ninaA Mutant Alleles, and DNA Sequencing—A *Calliphora* retinal cDNA library was screened with a radiolabeled *ninaA* cDNA (28) in 5 × SSC, 30% formamide, 0.25% SDS at 42 °C. Filters were washed in 1 × SSC, 0.25% SDS at 50 °C. Positive clones were isolated and subcloned into pBluescript II SK$^-$ (Stratagene).

The *ninaA* gene from each of the 70 independent mutants was amplified in two independent PCR reactions by the polymerase chain reaction (33). Oligonucleotides corresponding to nucleotides (−183)–(−163) (GCTGAAATCGACGTCTGCAG) and 816–835 (AATAAAGCTTATACTCCAAA) were used as amplification primers (28). Reactions were carried out in 10 mM Tris, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs, and 100 ng of each primer. Independently isolated subclones from different PCR reactions were sequenced for each mutant to eliminate potential errors occurring during PCR amplification. DNA sequencing was carried out by the dideoxy nucleotide termination method (34) using T7 sequencing (Pharmacia LKB Biotechnology Inc.).

Protein Gels and Western Blotting—Tissue homogenates were prepared by sonication in Laemmli buffer (35) containing 8 M urea. Retinal proteins were separated by electrophoresis in 10% SDS-polyacrylamide gels (35) and electroblotted onto nitrocellulose filters (36). The nitrocellulose was incubated with a monoclonal antibody directed to the C-terminal region of Rh1 (37), and the immunoreactive proteins were detected using ^{125}I-labeled Staph-A (ICN Radiochemicals). ninaA was detected with polyclonal antibodies generated against a bacteriophage T7 gene10-fusion protein that included residues 81–120 of *ninaA* (23, 29). Quantification was performed by scanning the ^{125}I-labeled Western blots on a PhosphorImager (Molecular Dynamics) and analyzing the results with Imagequant software (Molecular Dynamics). All Rh1 quantitations used five heads, and all ninaA quantitations used 10 heads per sample.

Overexpression, Purification, and Assays of CyP—Mutations were introduced into the rat CypA cDNA by synthesizing PCR primers containing mismatched nucleotides corresponding to the mutant sequence (C188Y, GAATTCTTAGAGTTGTCCATAGTCGGAGATGG; E110K, CACGCCATAATGGCACTGGTGGCAAGTCCATCTACGGAGAGAAATTTGAGGATAAGAACTTCATCC; G88D, GCTGCCATTATGGCGTGTGAAGTCACCATCCTGGCAC. The PCR products were introduced into an *Escherichia coli* expression vector containing the wild-type rat CyP-A cDNA (pET8C-rat) (38) and the entire gene sequenced after reconstruction. Expression plasmids were introduced into the strain pBL21 (pLysS) and proteins overexpressed by isopropyl-1-thio-β-D-galactopyranoside induction as described by (39).

Unlike wild-type CyP-A, the mutant proteins were insoluble when overexpressed, presumably due to incorporation into inclusion bodies (see Ref. 40). Fifty ml of induced cultures were centrifuged at 1500 × *g*, and the bacterial pellet was resuspended in 20 ml of 20 mM Tris, pH 7.8, 0.1 mg/ml phenylmethylsulfonyl fluoride. The cells were lysed by sonication (1 min on ice), the lysate centrifuged at 7000 × *g* and the pellet containing the insoluble protein was washed five times with 20 mM Tris, pH 7.8. The washed pellets were highly enriched for CyP-A protein. The CyP pellet was solubilized in 80 μl of 8 M urea, 20 mM Tris, pH 7.8. The CyP-A protein could then be purified to near homogeneity by chromatography on a Superose 12 column (Pharmacia) in 8 M urea, 20 mM Tris, pH 7.8. Since identical results were obtained with the solubilized pellet and the fast-protein liquid chromatography-purified protein, the latter purification step was not routinely used.

Wild-type and mutant CyP-A (40–200 μg in 20 μl) were renatured by diluting the urea-denatured overproduced proteins 35-fold with 20 mM Tris, pH 7.8. The samples were incubated at either 15 or 37 °C for 1 h. Following renaturation, the samples were spun for 15 min in an Eppendorf microcentrifuge at 4 °C, and the supernatant was used for prolyl isomerase assays. Prolyl *cis/trans* isomerase assays were conducted at 10 °C exactly as described by Lang and Schmidt (41).

RESULTS AND DISCUSSION

ninaA Mutations Define Important Residues in Cyclophilin—To identify amino acid residues in the ninaA protein that are important for its function *in vivo*, we set out to isolate novel *ninaA* alleles. Our screening strategy relied on the reduction of Rh1 rhodopsin levels characteristic of known *ninaA* mutants (26). This reduction leads to an attenuation of the DPP, an eye phenotype that can be readily scored in live flies under blue light illumination (31) (Fig. 1). We screened 700,000 EMS-mutagenized chromosomes for failure to complement a null *ninaA* allele (see "Experimental Procedures"). Seventy independent *ninaA* mutant alleles were recovered. Stocks were established from individual mutant lines, and genomic DNA from their *ninaA* gene was amplified by PCR and sequenced. Sixty-three of the 70 mutants showed single nucleotide changes (35 G→A; 14 C→T; 8 A→T; 2 G→C; 1 each C→A; T→A; T→G; T→C), and the remaining seven displayed small rearrangements, including duplications and deletions. The high bias toward G:C→A:T transitions reflects the mode of action of EMS (42; see "Experimental Procedures").

We have estimated the efficiency of this screen using the distribution of G→A and C→T transitions (17 single, 11 double, 2 triple, and 1 quadruple occurrence). These frequencies fit a Poisson distribution ($P = 0.15$) and indicate that we have achieved over 70% saturation. If instead, one calculates the screen's efficiency using the distribution of mutated glycine residues (encoded by GG*X* and thus a probable target of EMS) we have reached over 90% saturation (21 glycine changes distributed as 2 single, 3 double, 3 triple, and 1 quadruple occurrence). This calculation is supported by the observation that all termination codons that could have been generated by G:C→A:T transitions were recovered in this screen (7 out of 7). Since this screen was carried out to near saturation, information can be derived not only from those mutations that were isolated, but also from those that were not recovered. This latter class includes codons that could not have been hit given the mutagenic bias of EMS (*e.g.* a TTT codon), as well as those that did lead to an amino acid replacement but did not result in a mutant phenotype (either because of a conservative change that did not affect the protein or because that residue is not critical for function). Thus, for instance, no changes were recovered in the region encompassed by residues 2–44, yet they were likely mutated in this screen (see below).

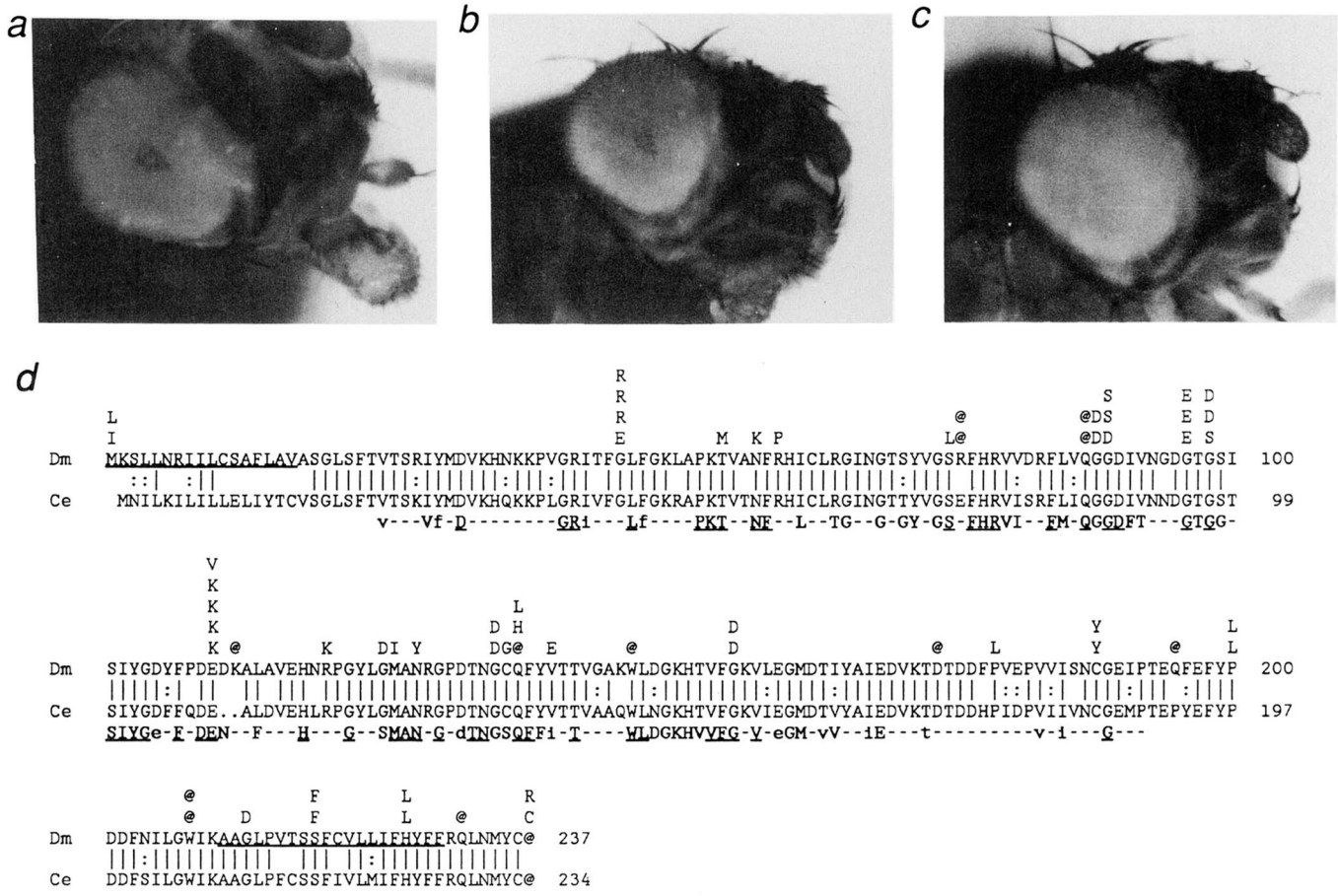

FIG. 1. **DPP and sequence comparison of wild-type and mutant ninaA.** *A*, shown is the DPP of a white-eyed wild-type control fly (***** in Table I). Each spot in the DPP represents superimposed images from multiple R1-6 photoreceptors from neighboring ommatidia (31). A reduction in rhodopsin levels in the R1-6 photoreceptor cells leads to structural alterations in the photoreceptors and changes in the interommatidial angle resulting in an attenuation of the DPP. *B*, weak *ninaA* mutant (***). *C*, null *ninaA* allele (*). *D*, Shown is a colinear alignment of the deduced amino acid sequence of the *Drosophila* (*Dm*) and *Calliphora* (*Ce*) ninaA proteins. *Vertical lines* indicate amino acid identities, and *dots* indicate conservative substitutions. *Underlined* are the N-terminal signal sequence and the C-terminal anchor domain of ninaA. Shown below the map is the consensus CyP "core" sequence. This consensus was derived from the alignment of human CyP-A (48), human CyP-B (49), mouse CyP-C (16), human CyP-2 and -3 (50), yeast CyP-1 and CyP-2 (51, 52), *Neurospora* CyP (53), *Drosophila* (28), and *Calliphora* ninaA and *Drosophila* CyP-1 (29). *Upper case letters* indicate high conservation (identity in 9 out of the 11 sequences), and *lower case letters* denote conservative substitutions. *Underlined* are residues which are conserved in all 11 sequences. 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. Shown above the alignment are the nature of the changes in 62 *ninaA* mutants. Nonsense mutations are denoted by @.

Fig. 1*D* shows an amino acid alignment of ninaA and the consensus cyclophilin sequence (see figure legend) with the location and nature of the amino acid change in 62 new *ninaA* alleles. These mutants show a range of phenotypes, both in DPP intensity and in the amount of Rh1 rhodopsin and ninaA levels (Table I and Fig. 2). We have also used the glycosylation state of Rh1 rhodopsin to distinguish between rhabdomeric (mature) and ER opsin (immature, see *arrow* in Fig. 2) (23). All of the strong *ninaA* alleles (*e.g.* G89D, G156D, Q195@, G213D) contain predominantly immature Rh1 opsin. This is consistent with the previous finding that Rh1 requires ninaA for exit from the ER (23).

The C-terminal Tail of ninaA Is Required for Its Function in Vivo—As a complementary strategy to define functionally important regions in the ninaA protein we isolated and sequenced the *ninaA* gene from a distantly related dipteran species, the blowfly *Calliphora erythrocephala* (Fig. 1*D*). ninaA is an integral membrane glycoprotein containing a cleavable N-terminal signal sequence and a hydrophobic C-terminal transmembrane domain (27, 29). It has the cyclophilin-homologous domain located in the lumen of the ER and a 7-amino acid C-terminal tail extending into the cytoplasm (29). The *Drosophila* and *Calliphora* proteins share 79% amino acid identity, with the most significant divergence in the N-terminal signal sequence (19% identity). Expectedly, the hydrophobic character of this domain is maintained. In contrast, the C-terminal hydrophobic membrane anchor domain, which is required for proper ninaA function (see truncations Q195@ and W208@, Table I), displays remarkable amino acid conservation (80% amino acid identity). Indeed, two additional mutations (S219F and H227L; Fig. 1*D*) introduced single amino acid changes that did not significantly alter the hydrophobic character of this region, yet they displayed strong ninaA mutant phenotypes (see Table I). These findings highlight a strong functional requirement for this transmembrane region, other than simply serving as a hydrophobic anchor domain. This region may be involved in the interaction of ninaA with rhodopsin, itself an integral membrane protein with over half of its structure buried in the lipid bilayer (43).

The last 7 residues of ninaA, which are thought to be located on the cytoplasmic face of the ER membrane, are also required

TABLE I

Mutagenesis summary

The table shows the relative levels of Rh1 rhodopsin, ninaA, and DPP in the different homozygous *ninaA* mutants. Amino acids are indicated by their single letter code and numbered according to Ref. 28. DEL and DPL refer to deletions and duplications, respectively; @ indicates a non-sense codon, and # indicates alleles that were tested as hemizygotes over a *ninaA* deficiency. Rhodopsin and ninaA levels were quantitated by Western blot analyses. Protein levels are presented relative to those of wild-type controls. Shown are averages of triplicate measurements. *, less than 7%; **, 7–25%; ***, 26–50%; ****, 51–75%, *****, greater than 75%. DPP recordings are presented as the average of four independent scoring sessions. The DPP range varies from 1–5; * represents a *ninaA* null mutant; and ***** represents a wild-type control. All measurements were carried out in flies raised at 25 °C. Some of the mutants are weak ninaA alleles and have deep pseudopupils that approach wild-type levels (*e.g.* G46E, T55M, G98S). These mutants were given a DPP score of five asterisks to reflect their weak phenotype. Note that many of these weak alleles still display dramatic reductions of ninaA levels (see text). Mutants are ordered according to their location in the primary sequence of the protein. Non-sense mutations preceding residue 195 were not quantitated and are not included. DEL[129–132]P replaces residues 129–132 with a proline.

Mutant	ninaA	Rh1	DPP
M1I	*	**	***
M1L	*	**	**
DEL[44–45]	**	*	*
G46R	**	***	**
G46E	**	*****	*****
T55M	**	****	*****
N58K	*	**	**
R60P	*	**	**
G88D	**	*	**
G89D	*	*	*
G89S	**	*	**
DPL[94–109]	**	****	****
G96E	*	*****	****
G98S	**	****	*****
G98D	**	*****	*****
E110K	*	****	****
E110V	*	****	*****
R120K	*****	***	*****
G125D	**	***	****
M126I	**	****	****
N128Y	**	***	***
DEL[129–132]P	**	***	****
G135D	**	**	***
C136G	**	***	****
Q137L	*	*****	*****
Q137H#	*	****	***
V140E	****	*****	*****
G156D	**	*	*
P179L#	*****	****	****
C188Y	**	****	***
Q195@	*	*	*
DEL[197–201]	**	*	*
P200L	*****	***	****
W208@	***	*	**
G213D	*	*	*
S219F	**	**	**
H227L	***	**	**
Q232@	*****	**	**
@238R	*	*	*

for ninaA's *in vivo* function. Three mutations that completely eliminate ninaA function were mapped to this region. These include a non-sense mutation that truncates the very last 6 residues (Q232@) and two missense mutations that eliminate the natural termination codon (Table I). It is likely that this region is involved in the interaction of ninaA with cytoplasmic components such as the intracellular targeting machinery (44)

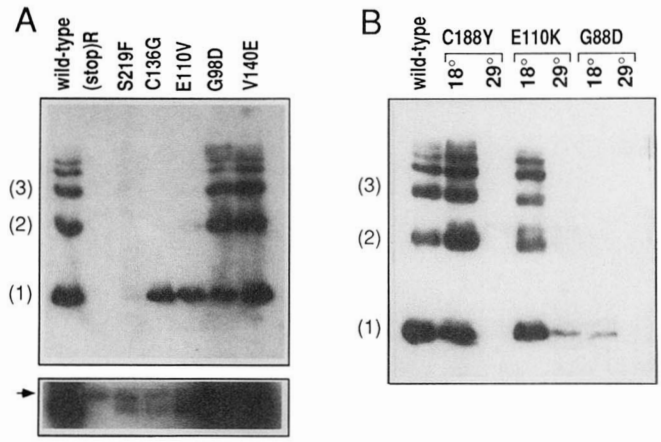

FIG. 2. **Rh1 opsin levels are defective in *ninaA* mutants.** *A*, the severity of the different *ninaA* alleles was determined by quantitative measurements of Rh1 rhodopsin levels. Shown is a Western blot containing protein extracts from wild-type controls (two heads per lane) and from an allelic series of *ninaA* mutants (strong to weak alleles, see Table I). *1*, *2*, and *3* refer to the monomer, dimer, and trimer forms of Rh1. *Drosophila* Rh1 opsin can be found in a mature, unglycosylated form or an immature core glycosylated form (23). Strong *ninaA* mutants contain mostly core glycosylated Rh1 stuck in the ER (23). The *lower panel* shows a 7-fold overexposure of the dimer region of the *upper blot* demonstrating the presence of predominantly immature opsin (*arrow*) in the two strongest alleles (*(stop)R* and *S219F*). *B*, Rh1 from *ninaA* temperature-sensitive mutants grown at 18 °C (permissive) and at 29 °C (restrictive). Each lane contains two heads. Note the dramatic reductions of Rh1 levels in the C188Y and E110K mutants. The G88D shows an example of a mutant with a severe reduction at permissive and restrictive temperatures. *1*, *2*, and *3* refer to the monomer, dimer, and trimer forms of Rh1. The levels of Rh1 in wild type controls are similar at 18 and 29 °C (data not shown).

and/or the cytoplasmic face of rhodopsin.

Mapping Mutations onto the Human Cyclophilin Crystal Structure—A very important aspect in the analysis of these mutants is to identify their position within the three-dimensional structure of the protein (*e.g.* are they clustered?). Although the structure of ninaA is not yet known, the structure of human cyclophilin-A (CyP-A), the major cytosolic receptor for CsA, has been recently determined both in an uncomplexed form (45) and complexed with a (*N*-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin)tetrapeptide (46). The protein has an eight-stranded antiparallel β-barrel structure, with the putative prolyl-isomerase substrate binding site and CsA binding site (47) on one face of the β-barrel. ninaA displays 42% amino acid identity with human CyP-A (28), suggesting a high degree of structural conservation. We have mapped the location of all *ninaA* missense mutations to the published structure of CyP-A (Fig. 3). Fourteen of the 20 amino acid changes shown in Fig. 3 fall in amino acid residues which are identical between *ninaA* and human CyP-A. Remarkably, most of the changes mapped to or near the β-barrel face that appears to be involved in CsA binding and prolylisomerase substrate binding. However, the overall footprint of the region defined by these mutations is much larger than that defined by the binding of the proline-containing tetrapeptide in the crystal structure. This most likely reflects the fact that the natural substrate(s) of CyPs are not small peptides but rather polypeptides, and is consistent with the notion that ninaA is specific for rhodopsin (23, 29), a larger substrate. Together, these results provide the strongest argument to date that the functionally relevant region of the molecule, *in vivo*, corresponds to and overlaps with the peptidyl-prolyl substrate-binding site.

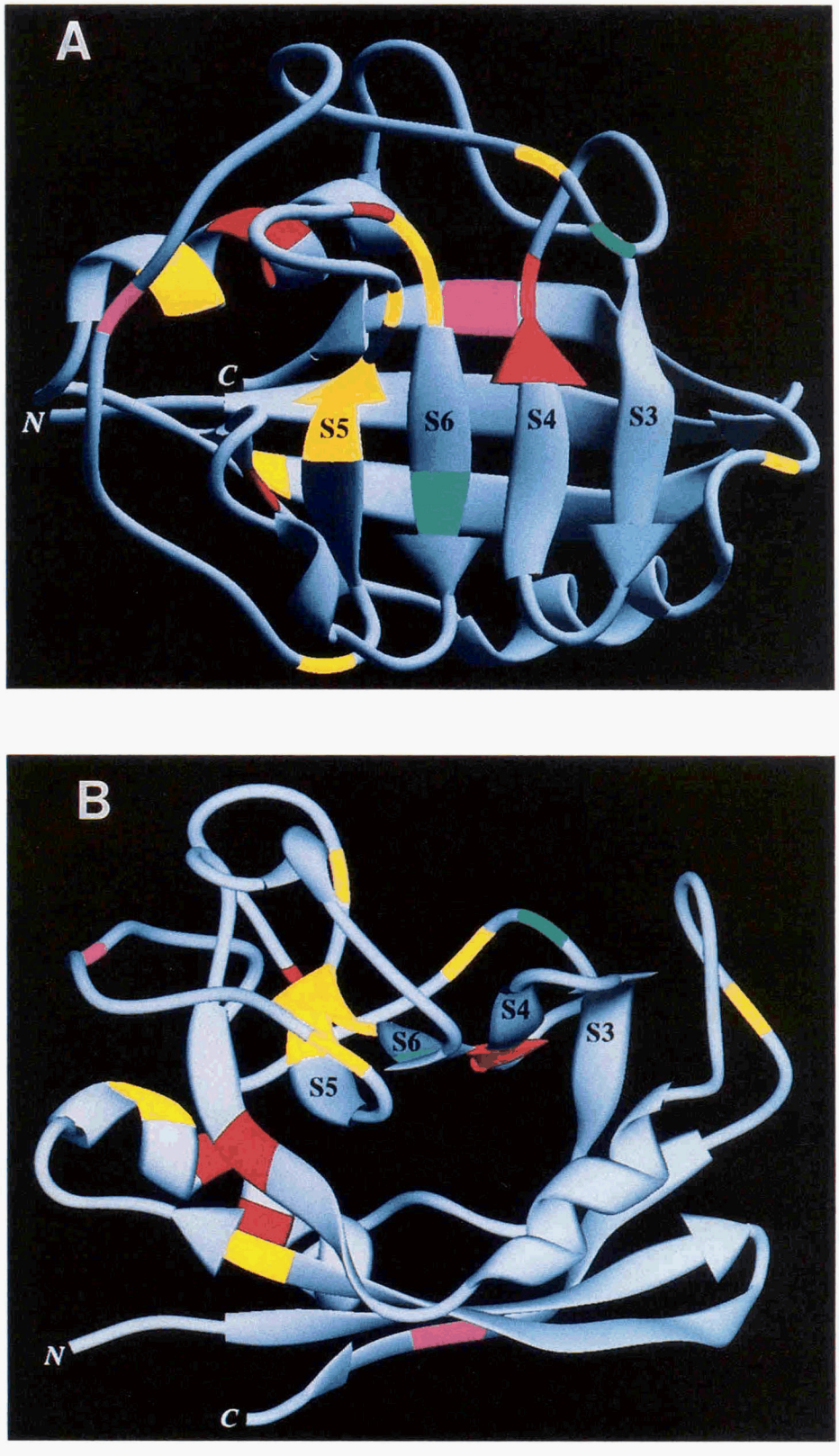

FIG. 3. **Mapping of ninaA changes to the crystal structure of human CyP-A.** *A*, ribbon diagram (54) illustrating the overall fold of human CyP-A (45, 46). Numbers indicate individual β-strands. The protein is an eight-stranded β-barrel. Shown are the location of 20 amino acid changes (see Table I). The severity of each allele has been color-coded: *red*, strong; *yellow*, moderate; and *green*, weak. The strongest allele was used when more than one mutation was identified at the same position (*e.g.* G46R and G46E). *Pink* indicates the location of the two temperature-sensitive alleles (C188Y and E110K). *B*, the figure in *A* has been rotated approximately 90° away from the viewer (toward the back plane of the page). Note the clustering of mutations between strands 4 and 6. This region has been implicated in CsA binding and peptidyl-prolyl substrate binding.

Among the mutants that did not fall within β-strands 4 to 6 are those that mapped to the C-terminal extension of ninaA, a region not found in the human CyP-A protein (see Fig. 1*D*) and a cluster of mutations in and around helix 1 (Fig. 3). It will be of interest to determine whether this latter class of mutants denotes a region required for the correct conformation of ninaA or whether they reflect a more specific aspect of its biological function.

Generation of Temperature-sensitive Cyclophilins—The isolation and characterization of temperature-sensitive (ts) *ninaA* alleles may provide a link between *in vivo* biological activity and biochemical function. In order to identify ts *ninaA* mutants, the DPP screen was carried out in flies reared at 29 °C, and then retested at 18 °C (see "Experimental Procedures"). We isolated two mutants that displayed a strong DPP at 18 °C (permissive temperature) but lacked a DPP at

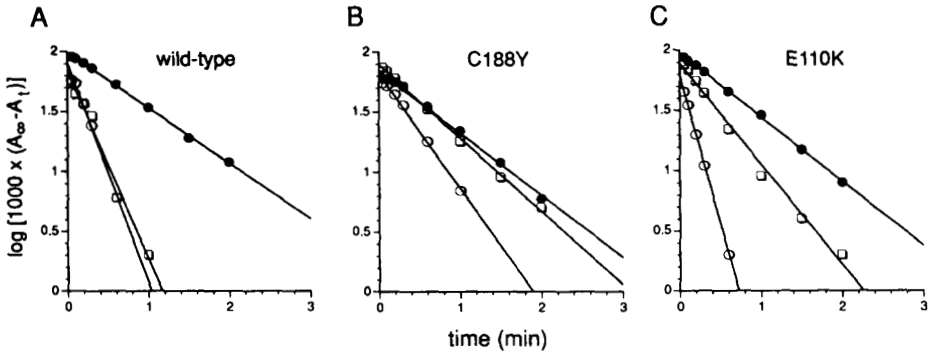

FIG. 4. ***ninaA* mutants define functionally important residues in cyclophilins.** Wild-type rat CyP-A or CyP-A containing missense mutations that produced temperature-sensitive *ninaA* alleles (C188Y and E110K) were assayed for peptidyl prolyl *cis/trans* isomerase activity after incubation at 15 or 37 °C (see "Experimental Procedures"). Prolyl isomerase activity was determined using a tetrapeptide substrate (succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide) (41). *cis/trans* isomerization of the alanine-proline bond is followed as an increase of absorbance at 390 nm. Shown are the log of absorbance changes as a function of time. *Closed circles*, uncatalyzed reaction; *open circles*, CyP-A incubated at 15 °C; *open squares*, CyP-A incubated at 37 °C. Note no changes in the activity of wild-type CyP-A following incubation at 15 or 37 °C. In contrast, the rat CyP-A^{C188Y} and CyP-A^{E110K} mutant proteins display dramatic temperature sensitivity. Identical results were obtained when the proteins were first refolded at 15 and then shifted to 37 °C (see "Experimental Procedures").

29 °C (restrictive temperature). Examination of rhodopsin levels in both mutants (C188Y and E110K) showed a marked temperature dependence (Fig. 2*B*). Mature rhodopsin levels are normal at the permissive temperature, but are dramatically reduced at the restrictive temperature. It is of interest that although rhodopsin levels are normal at the permissive temperature in these mutants (steady state measurements), ninaA itself is reduced 10–20-fold at the permissive temperature, consistent with a catalytic role for the protein.

To test whether the findings of this genetic screen can be extrapolated to other CyPs, the amino acid changes responsible for the two ts alleles (C188Y and E110K) were introduced into the homologous positions in the rat CyP-A gene (38). The mutant constructs were overexpressed in bacteria and the recombinant CyP-A proteins assayed for proline *cis/trans* isomerase activity after incubation at the permissive (15 °C) or restrictive (37 °C) temperature (see "Experimental Procedures"). As a control, we also overexpressed the wild-type rat CyP-A protein. As shown in Fig. 4, both mutant recombinant mammalian proteins exhibited temperature-sensitive prolyl *cis/trans* isomerase activity. In contrast, wild-type controls displayed normal isomerase activity at the permissive and restrictive temperatures (Fig. 4*A*). These findings highlight the structural similarity between different CyPs, and show that the results of this genetic screen may be generally applicable to other members of the CyP family.

Concluding Remarks—A very valuable aspect of this cyclophilin genetic screen is the identification of mutants based solely on their lack of biological activity. The requirement for *in vivo* function, irrespective of *in vitro* biochemical activities, allows for the unbiased dissection of biologically relevant regions of the molecule. In this context, it is worth noting that several of the mutants isolated in this screen could have not been predicted. For example, the requirement for the C-terminal transmembrane anchor domain (not only based on its structure but also on its specific primary sequence).

The results of this work provide the first dissection of structurally and functionally important regions of a CyP reflective of its normal cellular role *in vivo*. The overexpression and biochemical characterization of additional mutant proteins, based on *ninaA* alleles defined in this screen, should provide significant insight into the cellular function of CyPs.

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