Participation of Presenilin 2 in Apoptosis: Enhanced Basal Activity Conferred by an Alzheimer Mutation

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Overexpression of the familial Alzheimer's disease gene *Presenilin 2* (*PS2*) in nerve growth factor–differentiated PC12 cells increased apoptosis induced by trophic factor withdrawal or β-amyloid. Transfection of antisense *PS2* conferred protection against apoptosis induced by trophic withdrawal in nerve growth factor–differentiated or amyloid precursor protein–expressing PC12 cells. The apoptotic cell death induced by PS2 protein was sensitive to pertussis toxin, suggesting that heterotrimeric GTP–binding proteins are involved. A *PS2* mutation associated with familial Alzheimer's disease was found to generate a molecule with enhanced basal apoptotic activity. This gain of function might accelerate the process of neurodegeneration that occurs in Alzheimer's disease, leading to the earlier age of onset characteristic of familial Alzheimer's disease.

Molecular genetic studies have recently localized some mutations associated with familial Alzheimer's disease (FAD) to a gene named *Presenilin 2* (*PS2*) (*1*). The function of the protein PS2 is unknown, but its structure resembles that of heterotrimeric GTP-binding protein (G protein)–coupled receptors that have seven transmembrane domains. We recently showed that *ALG-3*, a truncated mouse homolog of *PS2*, rescues the mouse T cell hybridoma 3DO from T cell receptor–induced apoptosis (*2*). *ALG-3* is translated into a truncated PS2 polypeptide that acts as a dominant negative inhibitor of apoptosis (*3*). The involvement of PS2 in T cell receptor–triggered cell death prompted us to study whether PS2 is also required for neuronal cell death and whether mutations found associated with FAD affect PS2 activity.

To study the function of PS2 we used PC12 cells that had been grown in the presence of nerve growth factor (NGF) (100 ng/ml) for more than 2 weeks and were therefore dependent on NGF. Previous studies with neuronally differentiated PC12 cells (dPC12) have shown that withdrawal of trophic support induces apoptosis and that the peak of DNA fragmentation in this system, as shown by TUNEL staining, occurs 2 days after withdrawal of trophic support (*4*). In initial transfection studies we used sense, antisense, or ALG-3 constructs of mouse PS2 cDNA, which has been cloned (*3*). Parallel transfections with a β-galactosidase vector showed a transfection efficiency of 34 ± 6% (*5*). Both antisense PS2 and ALG-3 constructs decreased the amount of apoptosis in transfected dPC12 cells, as assessed by DNA laddering 18 hours after trophic factor withdrawal, whereas sense PS2 induced a small but reproducible increase (Fig. 1A). Quantitation of the changes using the TUNEL method showed that transfection of PS2 in the sense orientation induced some apoptosis even in the presence of serum and NGF (Fig. 1B, $P < 0.01$, analysis of variance). Withdrawal of trophic support for 48 hours increased the number of TUNEL-reactive nuclei in preparations of cells transfected with vector alone (Fig. 1B). Transfection with sense PS2

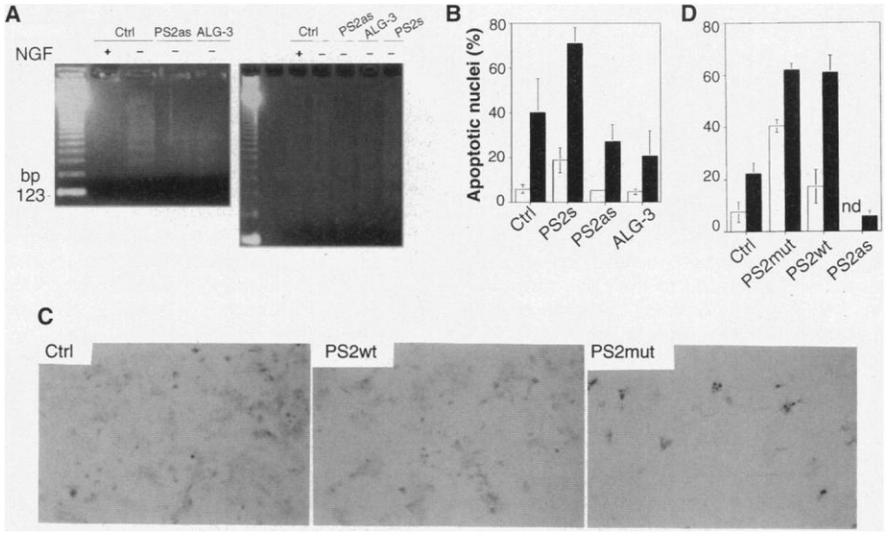

Fig. 1. Transient transfection studies show that PS2 participates in apoptosis induced by withdrawal of trophic support. Enhanced PS2 activity is conferred by an Alzheimer's mutation. (**A**) Endonucleolytic cleavage of dPC12 genomic DNA is normally induced by NGF withdrawal (*16*), but cleavage is reduced by transfection with antisense PS2 (PS2as) or ALG-3 and increased by overexpression of PS2 (PS2s) (results from two separate experiments are shown). Ctrl, vector-transfected cells. The left lane in each panel contains molecular size standards. (**B**) The amount of apoptosis was measured by the TUNEL method (*4, 17*) 96 hours after transfection. Six fields in two separate dishes were randomly selected, and the number of apoptotic nuclei as a percentage of the total nuclei were quantitated; all experiments were repeated at least three times independently. White bars, with NGF; black bars, without NGF and serum. (**C**) dPC12 cells transfected with vector (Ctrl) or wild-type human PS2 (PS2wt) show low levels of apoptosis, whereas cells transfected with N141I mutant human PS2 (PS2mut), in which Asn is mutated to Ile at position 141, show elevated levels of apoptosis and more cell detachment. (**D**) Quantitation of the results in (C) shows that PS2mut increases levels of apoptosis in cells grown under basal conditions fivefold over vector-transfected control cells. Both mutant and wild-type PS2 increase apoptosis under conditions of trophic deprivation to similar degrees. Apoptosis induced in vector-transfected dPC12 cells in this set of experiments was lower than that detected in the experiments shown in (B). White bars, with NGF; black bars, without NGF; nd, not detected.

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increased the amount of apoptosis in trophic factor–deprived cells by 53% compared with vector-transfected cells (Fig. 1B, $P < 0.01$). Transfection with antisense PS2 or ALG-3 reduced the number of apoptotic nuclei below that seen in the vector-transfected control by half (Fig. 1B, $P < 0.01$). Although antisense PS2 acts by reducing the amount of PS2 protein expressed (Fig. 2A), ALG-3 does not appear to decrease PS2 amounts (Fig. 2A), suggesting that it functions as a dominant negative inhibitor of PS2 action. These results indicate that PS2 directly participates in the biochemical pathway regulating apoptosis during trophic factor withdrawal.

Because mutations in PS2 are associated with FAD (*1*), we investigated whether such mutations influenced the role of PS2 in apoptotic processes. dPC12 cells were transfected with vector, FLAG-tagged human wild-type, FLAG-tagged N141I mutant (6), or mouse antisense constructs of PS2, and the amount of apoptosis was analyzed under basal or trophic-deprived conditions as described above. The human wild-type PS2 behaved similarly to the mouse PS2, being only slightly active under basal conditions (Fig. 1, C and D) but greatly enhancing apoptosis during trophic-factor withdrawal (Fig. 1D), whereas antisense PS2 prevented apoptosis (Fig. 1D). The N141I mutant form of PS2, however, behaved differently: it increased apoptosis under basal conditions fivefold ($P < 0.001$) (Fig. 1, C and D), whereas under trophic-deprived conditions it showed activity that was comparable with wild-type PS2 (Fig. 1D). This increased activity of the PS2 mutant was not due to differences in the amount of protein expression induced by the two vectors, because immunoblots and immunocytochemistry showed similar amounts of wild-type and mutant PS2 construct expression (Fig. 2, A and B). Moreover, the efficiencies of transfection, determined by scoring $FLAG^+$ cells, were comparable between the two vectors (41.7 ± 3.9 for wild-type PS2 and 39.1 ± 5.9 for mutant PS2). Titration of the mutant and wild-type genes also showed that the two constructs were equally active in enhancing apoptosis after trophic-factor withdrawal (Fig. 2C). Taken together, these experiments indicate that the FAD mutation confers strong constitutive apoptotic activity to PS2.

PS2 structurally resembles receptors containing seven transmembrane domains. A mutation in a similar position of the luteinizing hormone receptor also causes constitutive activation (*7*). This suggested that, like other similar proteins, PS2 might act through a heterotrimeric G protein. We therefore analyzed the sensitivity of PS2-mediated apoptosis to inhibition by pertussis toxin (PTX), an inhibitor of G_i and G_o proteins. We transfected dPC12 cells with human wild-type or mutant PS2, added PTX (100 ng/ml) 48 hours later, and quantitated the amount of apoptosis in trophic factor–stimulated or trophic factor–deprived cells as described above. PTX blocked the effects of mutant and wild-type PS2, although it did not alter the levels of apoptosis seen in the dPC12 cells transfected with the vector alone (Fig. 3). Thus, PS2 acts through a PTX-sensitive pathway, suggesting that G_i or G_o proteins are required for PS2 function.

It has recently been shown that overexpression of the amyloid precursor protein (APP) mutant APP^{Phe717}, dominantly inherited in chromosome 21 of individuals with FAD (8), induces apoptosis through a pathway sensitive to inhibition by PTX (9), which parallels the profile shown by PS2-induced apoptosis. However, the relation between these two proteins is unclear, and transfection of dPC12 cells with PS2 constructs does not appear to influence total APP protein amounts (Fig. 2A). Therefore, we sought to directly examine whether PS2 might play a role in apoptosis induced by APP^{Phe717}. Consistent with recent reports, we found that dPC12 cells stably transfected with various forms of APP showed increased levels of apoptosis after serum

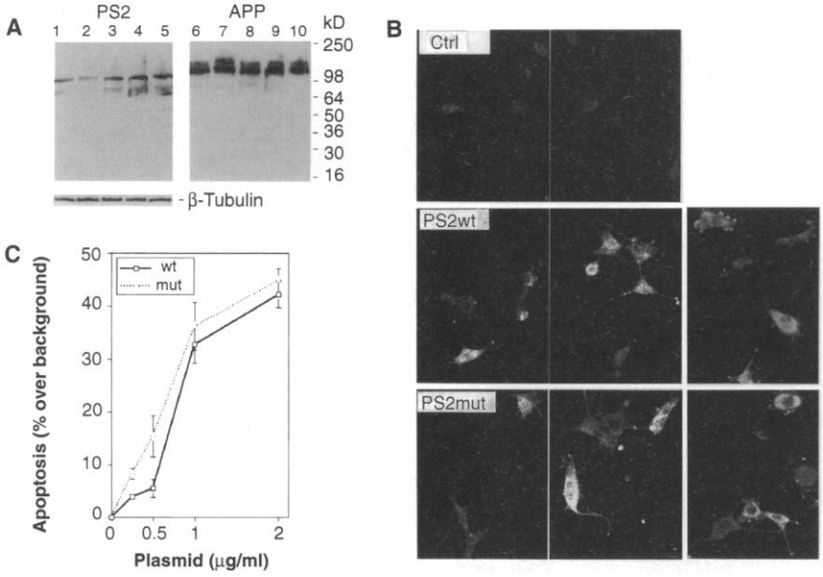


Fig. 2. (**A**) (Left) Immunoblot of transfected dPC12 cells with the antibody to PS2 (anti-PS2n) (*18*). The transfected wild-type (lane 4) or mutant (lane 5) PS2 are expressed to similar degrees, whereas transfection with antisense PS2 (lane 2) reduces the amount of PS2 below that in cells transfected with vector alone (lane 3). ALG-3 (lane 1) does not change expression levels of endogenous PS2. As previously noted (*6*), only a small fraction of PS2 runs according to its molecular size (~50 kD) because of aggregation typical of proteins having seven transmembrane domains. (Right) Transfection of PS2 constructs does not affect expression of amyloid precursor protein (APP) (constructs used for lanes 6 through 10 correspond to those used for lanes 1 through 5, respectively). The amounts of protein loaded were normalized with anti–β-tubulin (*18*). (**B**) Immunocytochemistry with antibody M2 to the FLAG epitope (*19*) shows that flagged PS2 wild-type (PS2wt) and mutant (PS2mut) constructs are expressed with similar efficiency in dPC12-transfected cells. Original magnification, ×20. (**C**) The potency of the PS2 vectors was assessed by transfecting dPC12 cells with various amounts of mutant or wild-type PS2, and the amount of apoptosis was quantitated by TUNEL (*17*). Both mutant and wild-type PS2 show similar potencies.

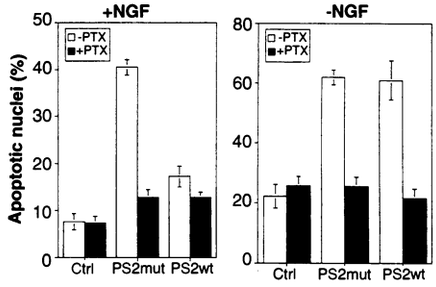


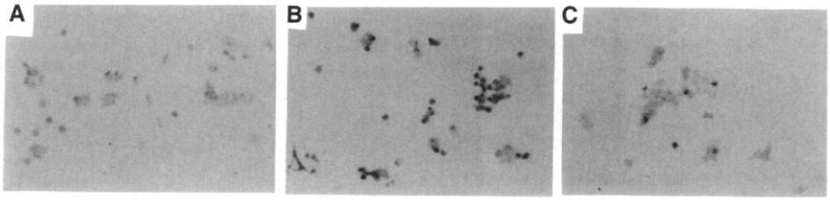
Fig. 3. PTX blocks apoptotic events mediated by PS2. dPC12 cells were transfected with vector (Ctrl), mutant, or wild-type PS2. The cells were treated on day 2 with PTX (*20*) and then cultured under basal conditions (+NGF) or trophic-deprived conditions (−NGF). At day 4 the cells were fixed and the amount of apoptosis was analyzed by TUNEL. PTX blocked apoptosis induced by the mutant PS2 under basal conditions. Trophic-deprived dPC12 cells showed some apoptosis even in the presence of PTX, but the greatly increased levels of apoptosis induced by mutant or wild-type PS2 were blocked by PTX.

withdrawal; APP^{Phe717} conferred the highest level of apoptosis compared with wild-type APP or APP^{Gly693} (Fig. 4D) (*10*). These PC12 cells were then transiently transfected with antisense PS2, vector control, or β-galactosidase constructs. The cells were serum-deprived 2 days after transfection, and apoptosis was analyzed by TUNEL after another 2 days. Transfection with antisense PS2 reduced the amount of apoptosis by more than 50% compared with cells transfected with vector alone (Fig. 4, A to D). This suggests that APP and PS2 share the same pathway of signal transduction.

The important role played by PS2 in cell death led us to examine whether it may participate in cell death mediated by β-amyloid peptide (Aβ) (*11*), which is a proteolytic product of APP. dPC12 cells were transfected with human wild-type or mutant PS2 or vector. Two days after transfection the cells were treated with aged Aβ(1–42) fragment for 2 days and then the amount of apoptosis was analyzed by TUNEL. Transfection of dPC12 cells with PS2 dramatically increased the amount of apoptosis induced by 10 μM Aβ (Fig. 4E). The ability of Aβ to activate the apoptotic pathway mediated by wild-type PS2 is similar to the results seen with trophic factor deprivation and supports the conclusion that PS2 actively participates in apoptosis. Moreover, constitutive activation of PS2, such as is seen with mutations associated with FAD, appears to increase the amount of apoptosis induced by Aβ (Fig. 4E).

In this study we have shown that PS2 participates in neuronal cell death induced by several stimuli and that a mutation associated with familial Alzheimer's disease generates a constitutively active molecule. The pathway of cell death mediated by both PS2 and APP (*9*), mutated in chromosome 21 of individuals with FAD (*8*), appears to involve heterotrimeric G proteins. These findings, as also shown by the ability of the antisense *PS2* to inhibit APP-mediated apoptosis, suggest that APP and PS2 are functionally related (*12*). The functional pathway used by PS2 may also be related to that of Fas, Fas-ligand, and related proteins, because ALG-3 can prevent T cell receptor–induced apoptosis by blocking the up-regulation of Fas-ligand (*2*). Because Fas-ligand shows a strong bystander effect, in which surrounding Fas-positive cells are induced to apoptose (*13*), a similar bystander effect may also be present with PS2-induced apoptosis. Whether the chromosome 14 FAD gene product PS1 (*14*), which shares substantial structural and amino acid similarity with PS2, subserves the same biochemical pathway remains to be determined.

Constitutive activation of PS2 increases the susceptibility of neurons to apoptotic stimuli that could sensitize neurons to the harmful insults of aging, such as free radical–mediated oxidation and neurotoxicity resulting from aggregated Aβ. The accelerated rate of neuronal cell death that occurs in the brains of Alzheimer patients may therefore result from the additive effects of activated apoptosis, aging, and Aβ toxicity. Interestingly, cell stress increases synthesis of APP and, depending on the particular stress, secretion of either APP or Aβ (*15*). Enhanced activation of PS2 might not only increase the tendency toward apoptosis, but by activating apoptotic signals, it may trigger a stress response and increase production of Aβ, adding to the toxic burden in the brains of Alzheimer patients and further accelerating the process of neurodegeneration.



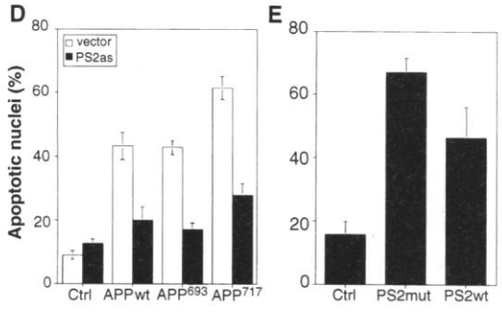


Fig. 4. PS2 participates in apoptosis induced by both APP^{Phe717} and by aged Aβ. dPC12 cells stably transfected with either pcDNA3 vector, wild-type (wt) APP^{751}, APP^{Glu693}, or APP^{Phe717} were transfected with vector or antisense mouse PS2, and apoptosis was analyzed by TUNEL on day 4 after 2 days of serum deprivation. (**A**) Control cells stably transfected with vector alone. All cells overexpressing APP showed increased levels of apoptosis after serum deprivation, but the cells transfected with the APP^{Phe717} construct (**B**) showed the most apoptosis. Transfection of the cells with antisense PS2 reduced the amount of apoptosis in all the cell lines overexpressing wild-type and mutant APP [(**C**) APP^{Phe717} plus antisense]. The transfection efficiency seen with the β-galactosidase vector in APP^{Phe717}-transfected cells was 36 ± 5%. (**D**) Quantitation of apoptosis after 2 days of trophic withdrawal. (**E**) Little apoptosis was seen in dPC12 cells after treatment for 2 days with aged Aβ(1–42) (*21*). However, treatment of dPC12 cells with Aβ did produce marked amounts of apoptosis in dPC12 cells transfected with mutant or wild-type PS2. Because mutant PS2 induces apoptosis by itself, parallel experiments without Aβ were also performed.

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16. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing NGF (100 ng/ml), 10% fetal bovine serum (FBS), 5% horse serum, and gentamycin sulfate (50 ng/ml). The cells were plated into 60-mm dishes and transfected the following day with lipofectamine (12 μl/ml) (Gibco-BRL) and plasmid (2 μg/ml). Trophic deprivation was accomplished by washing the cells twice with DMEM and then maintaining the cells in DMEM plus gentamycin sulfate (50 ng/ml). For extraction of apoptotic DNA fragmented in the internucleosomal spaces, cells were pelleted and suspended in 100 μl of 10 mM tris, 0.1 mM EDTA (pH 8.0) (TE). A volume of 200 μl of 2% NP-40 in TE was added, and the cells were agitated for 30 min at 4°C and spun for 15 min in a microfuge at 4°C. Supernatants were extracted with phenol and chloroform and precipitated in 300 mM sodium acetate (pH 5.1) and two volumes of ethanol. DNA was separated on a 1.5% agarose gel by electrophoresis.
17. For TUNEL analysis, the day after transfection cells

were treated with trypsin and transferred to chamber slides. A peroxidase-based TACS-TdT kit (Trevigen) was used according to the manufacturer's directions. After staining for apoptosis, three to six fields were chosen at random, and the numbers of total nuclei and apoptotic nuclei were counted. The experiments were repeated four to eight times with similar results.

18. For protein immunoblot analysis, the cells were harvested 3 days after transfection, lysed in radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitors aprotinin, pepstatin, and leupeptin (100 μg/ml each, Sigma). Cell lysates (10 μg) were denatured in modified 5× loading buffer (320 mM tris, pH 6.8, 50% glycerol, 0.5% bromophenol blue, 10% SDS, 100 mM dithiothreitol, and 8M urea) to reduce aggregation that typically occurs with proteins having seven transmembrane domains. After heating at 37°C for 45 min and then 75°C for 5 min, proteins were separated on a 4 to 20% polyacrylamide-SDS gradient gel, blotted onto nitrocellulose membranes (Gelman Science), and probed with either affinity-purified anti-PS2n (*3*), anti-APP (22C11, Boehringer Mannheim), or anti–β-tubulin (Boehringer Mannheim). Immunoblots were developed with the ECL System (Amersham).
19. For immunocytochemistry, 1 day after transfection cells were treated with trypsin and plated onto cover slips in a 24-well plate. The following day the cells were fixed for 20 min in 3% paraformaldehyde at 4°C, washed once with 1.5% glycine in PBS, twice with PBS, and blocked for 1 hour at 37°C with 10% bovine serum albumin in PBS. Samples were washed three times with 0.1% saponin in PBS (Sigma), incubated for 1 hour at 4°C with anti-FLAG (M2, Eastman Kodak), diluted to 5 μg/ml in PBS–0.1% saponin, and washed three more times with PBS–0.1% saponin. Cells were then stained for 1 hour at 4°C with fluorescein isothiocyanate–conjugated donkey antibody to mouse immunoglobulin G diluted 1:200 in PBS–0.1% saponin. After three washes, the cover slips were mounted onto a slide with fluoromount-g. Cells were viewed on a confocal Bio-Rad MRC1024 microscope at a ×20 magnification.
20. TUNEL studies were performed as described (*17*). PTX (Sigma, St. Louis, MO) was added once to achieve a final concentration of 100 ng/ml on day 2 and maintained in the medium until day 4.
21. For Aβ treatments the cells were maintained in DMEM containing 10% dialyzed FBS, 5% dialyzed horse serum, and gentamycin (50 ng/ml). The cells were treated with 10 μM Aβ(1–42) (generated from a 1 mM Aβ stock solution that had been aged 7 days at 37°C) starting on day 2 after the transfection. For all studies, the cells were fixed on day 4 and analyzed by TUNEL.
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A Cyclophilin Function in Hsp90-Dependent Signal Transduction

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Cpr6 and Cpr7, the *Saccharomyces cerevisiae* homologs of cyclophilin-40 (CyP-40), were shown to form complexes with Hsp90, a protein chaperone that functions in several signal transduction pathways. Deletion of *CPR7* caused severe growth defects when combined with mutations that decrease the amount of Hsp90 or Sti1, another component of the Hsp90 chaperone machinery. The activities of two heterologous Hsp90-dependent signal transducers expressed in yeast, glucocorticoid receptor and pp60$^{v\text{-}src}$ kinase, were adversely affected by *cpr7* null mutations. These results suggest that CyP-40 cyclophilins play a general role in Hsp90-dependent signal transduction pathways under normal growth conditions.

Immunophilins are ubiquitous and highly conserved. Nevertheless, with few notable exceptions, such as the participation of *Drosophila* cyclophilin ninaA in the maturation of rhodopsin (*1*) and the FKBP12-dependent regulation of Ca^{2+} flux by the inositol 1,4,5-trisphosphate receptor (*2*), little is known about the roles these molecules play in cell biology. Two *Saccharomyces cerevisiae* cyclophilins related to human CyP-40—Cpr6 and Cpr7—have been identified through their interactions with the transcriptional regulator Rpd3, a yeast homolog of histone deacetylase (*3*, *4*). Cpr6 and Cpr7 are the only CyP-40 homologs in *S. cerevisiae* (*4*).

Cpr6 was also identified as an Hsp90-associated protein (*5*). To determine if Cpr7 associates with Hsp90, we mixed glutathione-S-transferase (GST)–Cpr fusion proteins expressed in *Escherichia coli* with yeast cell lysates. Hsp90 was recovered on glutathione-affinity resin in association with both GST-Cpr6 and GST-Cpr7 but not with GST alone (Fig. 1, A and B). To determine if the interaction between Cpr6 or Cpr7 and Hsp90 requires other yeast proteins, we mixed GST-Cpr proteins with lysates of bacteria expressing Hsp90. Again, Hsp90 interacted with GST-Cpr6 and GST-Cpr7 but not with GST alone (Fig. 1C). The COOH-terminal halves of Cpr6 and Cpr7, which contain tetratricopeptide repeat (TPR) motifs thought to mediate protein-protein interactions (*4*), were sufficient for interaction with Hsp90 (Fig. 1C). Bacterially expressed GST-Cpr1, a *S. cerevisiae* cyclophilin that lacks a TPR motif (*6*), did not interact with Hsp90 (Fig. 1C). Thus, like the mammalian homolog CyP-40, Cpr6 and Cpr7 can interact directly with Hsp90 through their TPR-containing COOH-termini.

A functional relation between Cpr7 and Hsp90 was suggested through tests for genetic interaction. Because *cpr7*Δ cells display a slow-growth phenotype (*4*), we tested whether a decrease in Hsp90 concentrations would enhance this phenotype. *Saccharomyces cerevisiae* produces two nearly identical Hsp90 proteins (Hsc82 and Hsp82) that have the same functions but different patterns of expression (*7*). Deletion of *HSC82* reduces the cellular concentration of Hsp90 to approximately 1/10th of that in wild-type cells but does not impair growth below 37°C (*7*). Cells deleted for *HSC82* and *CPR7* exhibited a growth defect at 30°C that was more pronounced than that observed for *cpr7*Δ cells (Fig. 2A). Similar synthetic-enhancements of the *cpr7*Δ growth phenotype were observed in cells whose only source of Hsp90 was a temperature-sensitive allele, *hsp82*^{G170D} (*8*, *9*), and in cells deleted for *STI1*, which encodes another member of the Hsp90 chaperone complex (*5*, *10*) (Fig. 2B). In contrast, deletion of *CPR6* did not confer growth defects in either *hsc82*Δ or *sti1*Δ cells.

Hsp90 can act as a general chaperone in vitro to prevent aggregation of denatured proteins (*11*), but its functions in vivo seem to be focused on the maturation of various proteins that participate in gene regulation and signal transduction (*8*, *12*). Among the best characterized of these proteins is a subgroup of the steroid receptors (*13*–*15*). Several immunophilins are present in Hsp90 complexes, including the cyclophilin CyP-40 found in Hsp90::steroid receptor complexes (*5*, *13*, *15*, *16*). The role of these immunophilins in signal transduction, however, remains unclear.

The molecular components that mediate steroid receptor signal transduction in eukaryotes are highly conserved (*5*), and mammalian glucocorticoid receptor (GR) exhibits faithful hormone-dependent transcriptional activity in *S. cerevisiae* (*14*, *17*). Hsp90 is absolutely required for GR activity (*8*, *14*), whereas Sti1 is required for full receptor activity (*18*). We compared the activity of GR in isogenic (*19*) wild-type and cyclophilin-deleted (*cpr*Δ) cells with a *lacZ* GR reporter plasmid. With saturating concentrations of hormone (20 μM deoxy-

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