genetic approaches, such studies will lead to an understanding of molecular mechanisms for meiotic chromosomal events.

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5. Cells of a homothallic strain of *S. pombe*, h^{90} ade6-M210, cultured to mid-log phase in EMM2 (*3*) containing adenine sulfate (100 μg/ml) were transferred to EMM2 without nitrogen (EMM2–N) and incubated at 26°C to induce conjugation and meiosis. Live meiosis was first observed 6 hours after the nitrogen starvation. Cells were stained with Hoechst 33342 (0.5 μg/ml) in distilled water for 15 min at room temperature and then resuspended in EMM2-N. Live images of Hoechst 33342–stained cells were obtained on a cooled CCD with an exposure of 0.1 to 0.2 s under ultraviolet illumination of a mercury arc lamp. Stained cells were monitored after data collection to verify that spores were formed normally. An Olympus 60 ×/NA1.4 oil-immersion objective lens and a high-selectivity DAPI-Hoechst filter combination (Omega Optical, Brattleboro, VT, and Chroma Technology, Brattleboro, VT) were used in live analysis. The computer-controlled CCD microscope system is based on an Olympus inverted IMT-2 microscope equipped with a Peltier-cooled CCD (Photometrics, Tucson, AZ) with a 1340 by 1037 pixel CCD chip [Kodak KAF1400 (Rochester, NY)]. Details of the microscope system setup are the same as in (*20*) except that the MicroVAX (Digital Equipment, Boston, MA) was replaced by the Personal Iris 4D/35TG (Silicon Graphics, Mountain View, CA).
6. Haploid cells of h^{90} ade6-M210 were used for zygotic meiosis, and h^{+}/h^{-} ade6-M216/ade6-M210 diploid cells were used for azygotic meiosis. Cells were fixed with 3% formaldehyde (freshly prepared from paraformaldehyde) and 0.2% glutaraldehyde in PEM buffer [100 mM Pipes (pH 6.9), 1mM EGTA, 1mM $MgCl_2$] for 15 min at 26°C. After fixation, cells were permeabilized and treated with ribonuclease A as described (*7, 21*). The position of the SPB was determined by indirect immunofluorescence microscopy with an antibody to the fission yeast *Sad1* gene product, which is well characterized as a marker of the SPB in fission yeast mitotic cells (*7*), and a fluorescein-conjugated second antibody. Immunofluorescence labeling was carried out in PEM buffer as described in (*22*). After immunofluorescence labeling, cells were refixed with 3% formaldehyde and subjected to in situ hybridization. Plasmid pRS140 and cosmid cos212 were used as hybridization probes for centromeres and telomeres, respectively; pRS140 hybridizes to centromeric repeats in all three chromosomes (*23*), and cos212 hybridizes to both ends of chromosome I and II but not to chromosome III (*7*). DNA fragments were labeled with digoxigenin-dUTP by a terminal transferase reaction with the oligonucleotide tailing kit (Boehringer Mannheim, Indianapolis, IN). The heat-denatured DNA probe was hybridized to chromosomal DNA that had been denatured in 0.1 M NaOH. Hybridization was carried out in 50% formamide and 2× standard saline citrate at 37°C for 12 to 16 hours as described in (*21*). Hybridization signals were detected with the use of rhodamin-conjugated anti-digoxigenin antibody. Nuclear DNA was counterstained with DAPI (1 μg/ml). Triple-color fluorescent images of DAPI, fluorescein, and rhodamin were obtained on the computerized CCD microscope with an Olympus 100 ×/NA1.3 oil-immersion objective lens, high-selectivity filter combination and a triple-wavelength dichroic mirror (Omega Optical and Chroma Technology) (*20*).
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Requirement for the Yeast Gene *LON* in Intramitochondrial Proteolysis and Maintenance of Respiration

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The role of protein degradation in mitochondrial homeostasis was explored by cloning of a gene from *Saccharomyces cerevisiae* that encodes a protein resembling the adenosine triphosphate (ATP)–dependent bacterial protease Lon. The predicted yeast protein has a typical mitochondrial matrix–targeting sequence at its amino terminus. Yeast cells lacking a functional *LON* gene contained a nonfunctional mitochondrial genome, were respiratory-deficient, and lacked an ATP-dependent proteolytic activity present in the mitochondria of Lon^{+} cells. Lon^{-} cells were also impaired in their ability to catalyze the energy-dependent degradation of several mitochondrial matrix proteins and they accumulated electron-dense inclusions in their mitochondrial matrix.

The turnover of proteins within mitochondria remains poorly understood. Early work suggested that all mitochondrial proteins are degraded at the same rate (*1*), but later studies have shown that different mitochondrial proteins have different half-lives (*2*). As mitochondria appear to have evolved from endosymbiotic bacteria, selective degradation of mitochondrial proteins may be mediated by proteases similar to those of present-day bacteria. Indeed, Goldberg, Kužela, and their colleagues have found that the mitochondrial matrix contains an ATP-dependent protease resembling the Lon protease of bacteria (*3, 4*). Bacterial Lon plays an important role in the controlled degradation of short-lived or abnormal proteins (*5*).

To assess the physiological importance of the ATP-dependent mitochondrial protease, we sought to clone a *LON* gene homolog from *Saccharomyces cerevisiae*. We designed degenerate oligonucleotide primers (Fig. 1, arrows), exploiting the sequence conservation between different bacterial Lon proteases and the recently identified Lon homolog from humans (*6*). Using these primers in the polymerase chain reaction (PCR), we amplified a 1.2-kb fragment from both genomic yeast DNA and from a yeast complementary DNA (cDNA) library (*7*). The partial sequence of this fragment indicated that the potential protein product was markedly similar to the human and bacterial Lon proteins. This 1.2-kb fragment was used to clone the full-length gene from a yeast cDNA library (*8*). This gene, which we have designated *LON*, contains an open reading frame encoding a protein of 1133 amino acids.

The sequence of the yeast Lon protein is ~60% similar to the Lon proteases from *Bacillus brevis*, *Myxococcus xanthus*, and *Escherichia coli* and 65% similar to the human Lon homolog. Two regions are highly conserved among all homologs: the putative ATP-binding motifs between amino acids 633 and 730, and the region surround-

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ing a serine at the putative catalytic site (Fig. 1). In contrast to the bacterial Lon proteases, the predicted protein of *S. cerevisiae* has an NH_2-terminal extension that can potentially form a basic amphipathic helix, typical of a mitochondrial matrix–targeting signal. Yeast Lon differs from all other homologs by the presence of a highly charged insert between the ATP-binding and the catalytic domains (amino acids 839 to 883).

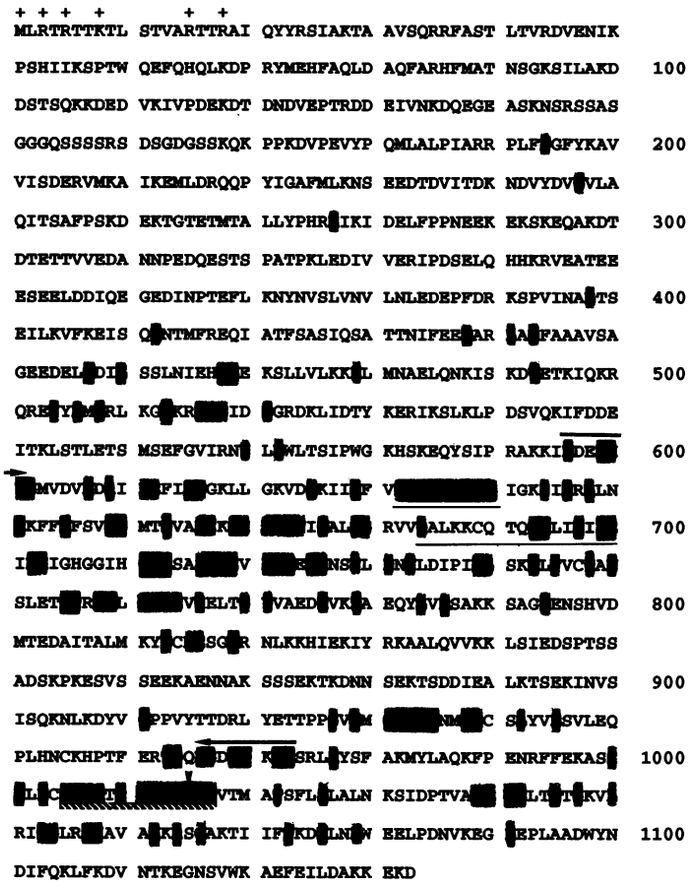

Fig. 1. Amino acid sequence encoded by the yeast *LON* gene. Basic residues in the putative matrix-targeting sequence of the yeast protein are marked by + signs. The amino acid residues identical among *S. cerevisiae, Homo sapiens, Myxococcus xanthus, Bacillus brevis*, and *Escherichia coli* are shaded. Arrows, regions to which degenerate oligonucleotide primers were made for the amplification of the yeast sequence by PCR; single underline, the ATP-binding motifs identified in the bacterial protease; hatched underline, the putative catalytic site; arrowhead, the serine residue postulated to be essential for the activity of the bacterial Lon protease.

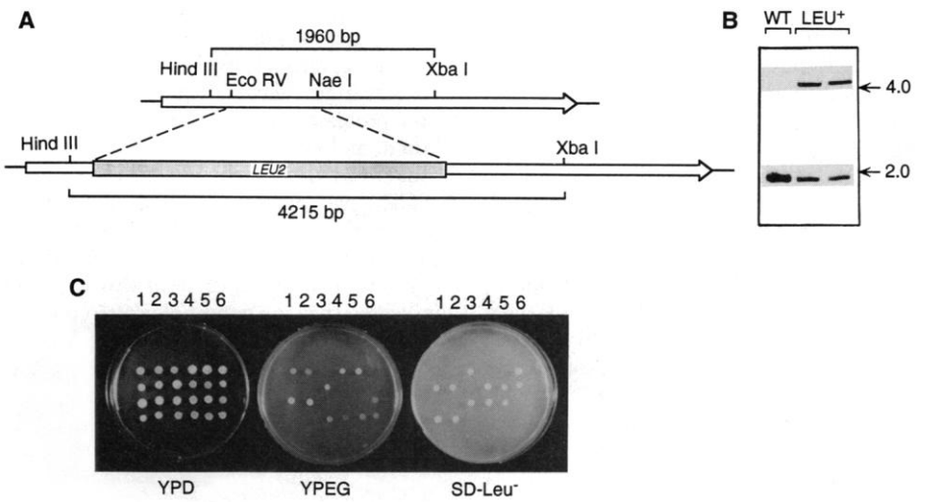

Fig. 2. Disruption of the *LON* gene in *S. cerevisiae* prevents growth on a nonfermentable carbon source. (**A**) The diploid yeast strain YKB5 was transformed to leucine prototrophy with a disrupted fragment of the yeast *LON* gene (open arrow) (*9*). (**B**) Southern blot analysis of genomic DNA from the diploid Leu$^+$ transformants (digested with the restriction enzymes Hind III and Xba I) confirmed the presence of one wild-type copy (1.96 kb) and one disrupted copy (4.2 kb) of the *LON* gene. Molecular sizes (in kilobases) are indicated at right. (**C**) Diploid transformants carrying a disrupted copy of the *LON* gene were sporulated. The spores from six tetrads were grown at 30°C on YPD plates (glucose-containing rich medium; left) and replica-plated onto YPEG (rich medium containing ethanol and glycerol; middle) or onto SD-leu$^-$ (glucose-containing synthetic medium lacking leucine; right).

We inactivated one of the two chromosomal *LON* genes in a diploid yeast strain by replacing an internal 745–base pair (bp) fragment with the *LEU2* gene (*9*) (Fig. 2, A and B). When the diploid transformants were sporulated and the resulting asci dissected onto glucose-containing rich medium, all four spores were viable (Fig. 2C, YPD). However, two spores of each tetrad were unable to grow on plates containing the nonfermentable carbon sources ethanol and glycerol, suggesting that they were respiratory deficient (Fig. 2C, YPEG). These two spores were always leucine prototrophs (Fig. 2C, SD-Leu$^-$), indicating that they had inherited the disrupted *LON* allele.

The inability of the haploid *LON* disruptants (*lon::LEU2*) to grow on nonfermentable carbon sources can be explained by aberrations in their mitochondrial DNA (mtDNA): polarographic measurements confirmed that the disruptants lacked cyanide-sensitive respiration; absorption difference spectra of their isolated mitochondria showed the absence of the mitochondrially encoded cytochromes b and aa_3; and mating of the disruptants with *LON* cells that totally lacked mtDNA failed to complement the respiratory deficiency (*10*). These combined characteristics indicate that the *lon::LEU2* disruptants belong to a class of mutants that are respiratory-deficient because of the absence of mtDNA (ρ^0) or extensive deletions in mtDNA (ρ^-) (*11*). Staining of the *lon::LEU2* cells with the fluorescent DNA stain 4,6-diamino-2-phenylindole (DAPI) indicated that mtDNA was still present (*10*). Thus, disruption of the yeast *LON* gene results in a ρ^- phenotype and the loss of respiratory function.

We tested whether *lon::LEU2* cells had lost the ATP-dependent proteolytic activity previously identified in the mitochondrial matrix (*4, 12*). We first converted the *LON* cells to ρ^- mutants by acriflavin mutagenesis (*LON* ρ^-) (*13*) to ensure that the physiological states of the *LON* and *lon::LEU2* cells were as similar as possible. Mitochondrial matrix from *LON* and *lon::LEU2* cells was partially purified on Q-Sepharose and assayed for the ability to degrade ^{125}I-labeled casein to acid-soluble peptides in an ATP-dependent fashion (*14*). The proteolytic activity of the wild-type fraction was stimulated twofold by 2 mM ATP. The activity of the mutant fraction was not stimulated by ATP and was similar to the ATP-independent activity found in the wild-type matrix fraction (*14*). This difference was not caused

by unequal recovery of chromatographed matrix proteins because the respective column eluates contained comparable amounts of protein and very similar protein composition as judged by SDS–polyacrylamide gel electrophoresis (PAGE). Thus, loss of a functional *LON* gene is accompanied by the significant loss of an ATP-dependent proteolytic activity in the mitochondrial matrix.

To determine whether the *LON* gene product controls selective mitochondrial protein turnover in vivo, we examined the stability of various mitochondrial proteins in *LON* ρ^- and *lon::LEU2* cells. After cells were grown in galactose-containing rich medium at 30°C, the levels of several matrix proteins were measured by immunoblotting before and after a 1-hour chase at 39°C in the presence of cycloheximide (CHX), an inhibitor of cytoplasmic protein synthesis. Two matrix proteins proved to be unstable in *LON* cells: the β subunit of the general matrix peptidase (MPPβ) (Fig. 3, hatched bars) and the F_1–adenosine triphosphatase (ATPase) β subunit ($F_1\beta$) (Fig. 3, Gal and Glu with hatched bars). In *LON* cells, 60% of MPPβ was degraded during the 1-hour chase; in the *lon::LEU2* disruptants, degradation of this protein was insignificant (Fig. 3, black bars). Degradation of mitochondrial proteins in *LON* cells appeared to be selective: Other matrix proteins such as aconitase, α-ketoglutarate dehydrogenase, and mitochondrial hsp70, as well as the mitochondrial inner membrane enzyme cytochrome c_1, remained stable under these conditions (*10*).

Lon-dependent degradation of matrix proteins is energy-dependent in vivo. When galactose-grown *LON* cells were chased in the presence of glucose and CHX, about 50% of $F_1\beta$ was degraded. In contrast, when cells were chased in the presence of 2-deoxyglucose and azide (to deplete intracellular ATP), degradation was only 10% (Fig. 3). The stability of $F_1\beta$ in the energy-depleted *LON* cells was thus comparable to that in energy-sufficient *lon::LEU2* cells (Fig. 3). Taken together, these results suggest that the *LON* gene encodes an ATP-dependent protease that mediates the selective turnover of mitochondrial matrix proteins.

Loss of the *LON*-encoded function markedly affects the morphology of mitochondria. Thin-section electron microscopy of *LON* respiratory-competent (ρ^+) cells revealed typical mitochondrial profiles with numerous cristae (Fig. 4A). In *LON* cells containing mtDNA with internal deletions (ρ^- mutants), cristae were infrequent; the inner membrane was either undiscernible or appeared to transect the mitochondrial profiles (Fig. 4B), or formed concentric lamellae (*15*). The mitochondria of *lon::LEU2* ρ^- cells in many ways resembled those of *LON* ρ^- cells. However, their mitochondria often appeared more irregular and their matrix space was filled with electron-dense inclusion bodies (Fig. 4, C and D). Such inclusions were much less frequent and less extensive in *LON* ρ^- cells. The chemical composition of these electron-dense deposits is unknown, but it is tempting to speculate that they represent aggregated mitochondrial proteins that accumulate in the absence of Lon-mediated proteolysis.

The results presented here begin to reveal the fundamental importance of the Lon protease for mitochondrial function. We do not know whether the *LON*-encoded function is required directly or indirectly for maintaining an intact mitochondrial genome. Additional unanswered questions are how the protease selects its substrates, whether its function in vivo requires other protein components, and whether its own synthesis in the cytosol is induced by abnormal proteins inside mitochondria. The molecular cloning of the *LON* gene from yeast and the availability of a Lon null mutant will help to answer some of these questions.

Note added in proof: Van Dyck *et al.* (*16*) report the cloning of the *PIM1* gene in *S. cerevisiae* that is identical with the *LON* gene described in our study.

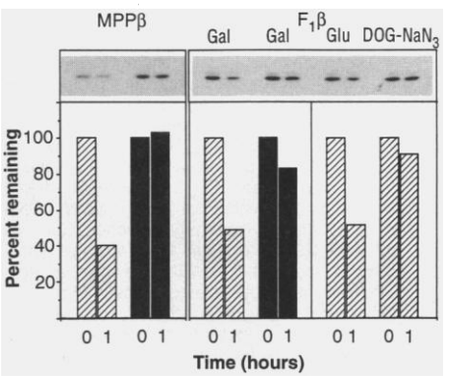

Fig. 3. ATP-dependent proteolysis in vivo is controlled by the *LON* gene. (**Left**) Respiratory-deficient *LON* (hatched bars) and *lon::LEU2* (black bars) cells were grown at 30°C to mid-log phase [absorbance at 600 nm (A_{600}) ~1.0] in YPGal. A sample was withdrawn (t = 0) and the culture was shifted to 39°C. After 30 min, cycloheximide (CHX) was added to 100 μg/ml; 1 hour after addition of CHX, another sample was withdrawn (t = 1). The elevated temperature was chosen to enhance intracellular protein degradation. After each time point the cells were harvested; their proteins were extracted (*17*) and analyzed by SDS-PAGE and immunoblotting for the β subunit of the general matrix peptidase (MPPβ). (**Right**) Respiratory-deficient *LON* (hatched bars) and *lon::LEU2* (black bars) cells were grown to an A_{600} of 1.0 in YPGal at 30°C. The cultures were then shifted to 39°C. After 30 min, a sample was withdrawn (t = 0). The *lon::LEU2* sample was directly supplemented with CHX, and the *LON* culture was divided into three equal portions. The first portion was directly supplemented with CHX; the second was centrifuged and the cells were resuspended to the original volume with YP containing 5% glucose and CHX; the third was resuspended in 5% 2-deoxyglucose (DOG), 1 mM NaN_3, and CHX. The samples were incubated for 1 hour at 39°C, after which another time point was taken (t = 1). Cellular proteins were extracted and the β subunit of F_1-ATPase ($F_1\beta$) was visualized by immunoblotting.

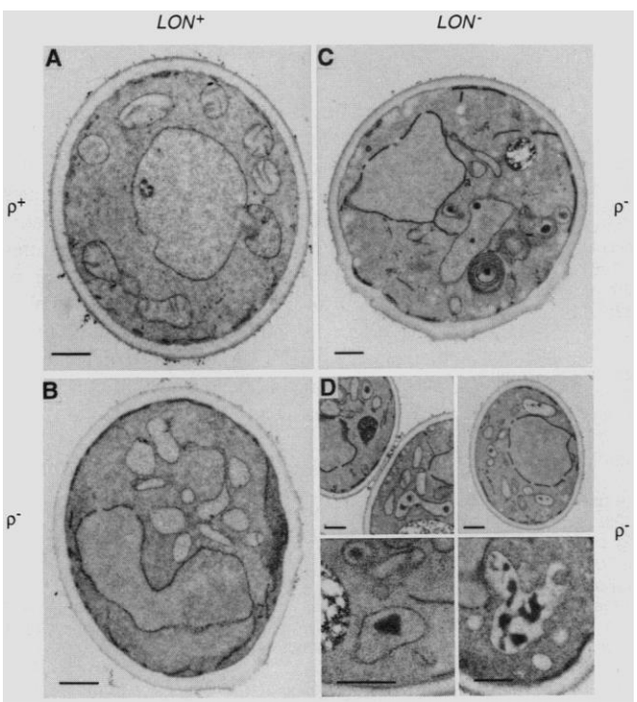

Fig. 4. *lon::LEU2* cells accumulate electron-dense inclusions in their mitochondrial matrix. (**A**) *LON* ρ^+ cells, (**B**) *LON* ρ^- cells, and (**C** and **D**) *lon::LEU2* ρ^- cells were grown in YPGal medium at 30°C to mid- or late log phase (A_{600} = 1 to 3), mixed with an equal volume of 4% glutaraldehyde, 4% formaldehyde, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 M sodium cacodylate (pH 6.8), and incubated on ice for 60 min. The cells were washed, incubated with 1% $KMnO_4$ for 60 min, washed again, incubated with 2% uranyl acetate for 60 min, dehydrated, infiltrated with Spurr's low viscosity resin, sectioned at 40 to 50 nm, and contrasted with Reynold's lead citrate. Bars, 0.5 μm.

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7. Two degenerate oligonucleotide primers were synthesized: 5′-GTI(T/C)TIGAIGA(A/G)GA(T/C)-CA(T/C)TA(T/C)GG-3′ and 5′-GA(T/C)TC(T/C)-TTCATIACITCICCIA(A/G)(T/C)TG-3′. The first is a 32-fold degenerate 23-nucleotide oligomer in the sense direction, corresponding to amino acids 490 to 497 of the human Lon protein. The second is a 16-fold degenerate 26-nucleotide oligomer in the antisense direction, corresponding to amino acids 809 to 817 of the human Lon protein. PCR was carried out in a total volume of 100 μl, in the presence of 2.5 mM $MgCl_2$, 1 to 2 μM primers, and either 0.4 μg of a yeast cDNA library [H. Liu, J. Krizek, A. Bretscher, *Genetics* **132**, 665 (1992)] or DNA from a single yeast colony. A fragment of ~1.2 kb was amplified from both templates, gel-isolated, ligated into a TA cloning vector (Invitrogen, San Diego, CA), and sequenced by the dideoxy method.
8. The 1.2-kb PCR fragment was ^{32}P-labeled by random priming to a specific activity of $\sim 4 \times 10^8$ counts per minute (cpm) per microgram of DNA. This probe was used to screen 50,000 colonies of a cDNA library. Positive colonies were purified by rescreening.
9. A 745-bp Eco RV–Nae I fragment from within the *LON* gene was replaced by a blunted 3.0-kb Bgl II fragment carrying the yeast *LEU2* gene. A Hind III–Xba I fragment containing the *LEU2* gene flanked by 0.2 kb and 1.0 kb of the *LON* gene was purified by gel electrophoresis and used to transform the diploid yeast strain YKB5 (*MATa*/*MATα ura3/ura3, his4/his4, leu2/leu2, lys2/LYS2, ade2/ADE2*) to leucine prototrophy [D. Gietz, A. St. Jean, R. A. Woods, R. H. Schiestl, *Nucleic Acids Res.* **20**, 1425 (1992)]. Chromosomal DNA from transformants was digested with Hind III and Xba I and probed with a random-primed fluorescently labeled Hind III–Xba I fragment by Southern (DNA) blotting (ECL Random Prime Labelling System, Amersham, Buckinghamshire, England).
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13. *LON* cells were converted to ρ^- mutants by growth in the presence of acriflavin (20 μg/ml). The presence of mtDNA was ascertained by staining of the cells with DAPI, and respiratory deficiency by inability to grow on rich medium containing ethanol and glycerol as carbon sources.
14. *LON* ρ^- and *lon::LEU2* cells were grown in YPGal (1% yeast extract, 2% peptone, 2% galactose) at 30°C and harvested during log phase growth. Mitochondria were isolated as described [G. Daum, S. M. Gasser, G. Schatz, *J. Biol. Chem.* **257**, 13075 (1982)], except that the phenylmethylsulfonyl fluoride in the isolation medium was replaced by 1 mM each of TLCK and TPCK. The mitochondria were converted to mitoplasts by osmotic shock [B. S. Glick *et al.*, *Cell* **69**, 809 (1992)]. Matrix contents were released from mitoplasts by sonication on ice and centrifuged at 100,000*g* for 5 min at 4°C. The supernatant was loaded onto a Q-Sepharose Fast Flow ion exchange column (0.5 ml bed volume) equilibrated with buffer A [20 mM tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerin). Casein (100 μg) was radiolabeled with 100 μCi of carrier-free $Na^{125}I$ by the chloramine-T method [F. C. Greenwood, W. M. Hunter, J. S. Glover, *Biochem. J.* **89**, 114 (1963)]; the specific radioactivity of the radioactively labeled casein was adjusted with unlabeled casein to 30 μCi per milligram of casein. Proteolysis was assayed as described by Y. Katayama, S. Gottesman, and M. R. Maurizi [*J. Biol. Chem.* **262**, 4477 (1987)]. In the presence of 2 mM ATP, matrix fractionated from *LON* mitoplasts released 12,000 ± 290 cpm of acid-soluble ^{125}I-peptides; in the absence of added ATP, 5700 ± 157 cpm were released. With matrix fractionated from *lon::LEU2* mitoplasts, the corresponding values were 5600 ± 1184 cpm and 4254 ± 192 cpm, respectively.
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18. We thank R. D. Klausner for support during the cloning of the yeast *LON* gene; S. Gottesman, M. M. Gottesman, and M. Maurizi for transmitting data before publication and for reviewing this manuscript; and A. Bretscher (Cornell University, Ithaca, NY) for providing colonies of a yeast cDNA library. We are grateful to U. Sauder and M. Duerrenberger (Biozentrum) for technical assistance with the electron microscopy. Supported by grant 3-26189.89 to G.S. from the Swiss National Science Foundation, by a grant from the European Community Human Capital and Mobility Program, and by a postdoctoral fellowship to C.K.S. from the Damon Runyon–Walter Winchell Cancer Research Foundation.

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Soluble β-Amyloid Induction of Alzheimer's Phenotype for Human Fibroblast K^+ Channels

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Although β-amyloid is the main constituent of neurite plaques and may play a role in the pathophysiology of Alzheimer's disease, mechanisms by which soluble β-amyloid might produce early symptoms such as memory loss before diffuse plaque deposition have not been implicated. Treatment of fibroblasts with β-amyloid (10 nM) induced the same potassium channel dysfunction previously shown to occur specifically in fibroblasts from patients with Alzheimer's disease—namely, the absence of a 113-picosiemen potassium channel. A tetraethylammonium-induced increase of intracellular concentrations of calcium, $[Ca^{2+}]_i$, a response that depends on functional 113-picosiemen potassium channels, was also eliminated or markedly reduced by 10 nM β-amyloid. Increased $[Ca^{2+}]_i$ induced by high concentrations of extracellular potassium and 166-picosiemen potassium channels were unaffected by 10 nM β-amyloid. In Alzheimer's disease, then, β-amyloid might alter potassium channels and thus impair neuronal function to produce symptoms such as memory loss by a means other than plaque formation.

The protein β-amyloid (βAP) is implicated as a key factor in the pathophysiology of Alzheimer's disease (AD) (*1*, *2*). It is the main component of the neurite plaques which, together with the neurofibrillary tangles, constitute the neuropathological features that confirm AD (*3*). Mutations of the amyloid precursor protein (APP), from which βAP originates, are present in a limited number of AD cases (1 to 5%) (*4*), and altered APP processing has been proposed to result in the generation of amyloidogenic protein fragments (*5*).

Soluble βAP can be the product of normal proteolytic processing (*2*, *6*), whereas an excess of βAP is released from a mutant APP (*7*). Proposed mechanisms for βAP's deleterious effects include direct neurotoxicity (*8*, *9*), induction of calcium metabolism derangements leading to enhanced excitotoxicity (*10*), membrane permeability enhancement (*11*), and formation of Ca^{2+} channels that would lead to abnormally high cytosolic Ca^{2+} concentrations and subsequently cell death (*12*). Nevertheless, mechanisms by which βAP causes symptoms such as memory loss without plaque formation have not been implicated.

A 113-pS tetraethylammonium (TEA)-sensitive K^+ channel is functionally absent in fibroblasts from AD patients (*13*). As a consequence, the increase in $[Ca^{2+}]_i$ observed after depolarization induced by TEA in fibroblasts derived from control individuals [including fibroblasts from young individuals (YC), age-matched individuals (AC), and

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