Promotion of Mitochondrial Membrane Complex Assembly by a Proteolytically Inactive Yeast Lon

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Afg3p and Rca1p are adenosine triphosphate (ATP)–dependent metalloproteases in yeast mitochondria. Cells lacking both proteins exhibit defects in respiration-dependent growth, degradation of mitochondrially synthesized proteins, and assembly of inner-membrane complexes. Defects in growth and protein assembly, but not in degradation, were suppressed by overproduction of yeast mitochondrial Lon, an ATP-dependent serine protease. Suppression by Lon was enhanced by inactivation of the proteolytic site and was prevented by mutation of the ATP-binding site. It is suggested that the mitochondrial proteases Lon, Afg3p, and Rca1p can also serve a chaperone-like function in the assembly of mitochondrial protein complexes.

Chaperones that mediate the folding of soluble proteins have been identified, and their molecular mechanisms of action continue to be characterized. Less is known, however, about the assisted folding of membrane proteins and the assembly of multisubunit complexes. In the yeast *Saccharomyces cerevisiae*, several proteins have been identified that are involved in the assembly of hetero-oligomeric complexes of the mitochondrial inner membrane. Two of these proteins, Afg3p (also termed Yta10p) (*1*) and Rca1p (also termed Yta12p) (*2*, *3*), are homologous to bacterial FtsH (*4*) and belong to the family of AAA proteins [AAA denotes adenosine triphosphatases (ATPases) associated with diverse cellular activities] (*5*). Both Afg3p and Rca1p contain specialized forms of the Walker Box ATP-binding motif (*6*) and a HEXXH motif (*7*) found in Zn^{2+}-dependent proteases (*8*). In Afg3p, both motifs are found at the COOH-terminus that appears to be exposed to the matrix (*9*).

Deletion of the genes *AFG3* or *RCA1*, or both, prevents growth on nonfermentable carbon sources (*1*, *3*) and abrogates the degradation of mitochondrially synthesized proteins (*9*) and the assembly of cytochrome c oxidase (Cox) and F_1F_0 ATPase (*10*). Three multicopy suppressors of the *afg3* or *rca1* growth defect have been identified (*11*): Oxa1p, which is required for the assembly of Cox and F_1F_0 ATPase (*12*); Mba1p, which has no defined function (*13*); and yeast Lon (also termed Pim1p), which encodes an ATP-dependent mitochondrial protease (*14*, *15*).

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Overproduction of wild-type Lon restored growth of the double mutant, *afg3 rca1*, on the nonfermentable carbon sources ethanol and glycerol (*16*) (Fig. 1). To address whether this suppression of the *afg3 rca1* growth defect depends on the proteolytic activity of Lon or on some other function, we tested variants of Lon in which the active-site serine or the ATP-binding motif had been modified by site-directed mutagenesis (*17*, *18*) (Fig. 1). Strong suppression was observed with a proteolytically inactive Lon in which the conserved Ser^{1040} at the active site had been mutated to alanine (LON S1040A); such a mutation abolishes proteolytic activity of Lon from *Escherichia coli* (*19*) (Fig. 1). In contrast, no suppression by Lon was observed when the conserved Lys^{638} within the ATP-binding motif (GPPGVGKT) (*7*) was mutated to asparagine (LON K638N) (*17*) (Fig. 1). Studies with various ATP-binding proteins have indicated that this lysine residue interacts directly with the β- and γ-phosphates of bound nucleotide (*20*). Suppression by the proteolytically inactive Lon was also abolished by mutating the ATP-binding site (LON K638N/S1040A). Thus, a function distinct from Lon's proteolytic activity appeared to be responsible for restored growth, and this function required an intact ATP-binding site.

To confirm that the Lon S1040A mutant was indeed proteolytically inactive, we compared the ability of wild-type Lon and of Lon S1040A to degrade a protein substrate in vitro and in vivo. Lon proteins that had six histidine residues fused to their COOH-terminus were isolated on Ni^{2+}-agarose, and their ability to degrade ^{125}I-labeled casein in an ATP-dependent fashion was measured in vitro (Fig. 2A). Wild-type Lon catalyzed the ATP-dependent degradation of casein, whereas Lon S1040A was inactive. To assess the proteolytic activity of the wild-type and mutant Lon proteins in vivo, we measured the steady-state levels of the α and β subunit of the F_1 ATPase in Lon-deficient yeast cells (Δlon) and in cells overproducing wild-type Lon (Δlon + LON) or mutant Lon (Δlon + LON S1040A) (Fig. 2B). In cells overproducing wild-type Lon, steady-state levels of $F_1\alpha$ and $F_1\beta$ were decreased to 41% and 18% of the levels in the Δlon mutant. In contrast, cells overproducing Lon S1040A had the same steady-state levels of both F_1 subunits as did Δlon cells. Thus, as expected, the S1040A mutation abolished the proteolytic activity of Lon.

To examine whether overproduction of wild-type or proteolytically inactive Lon could alleviate the degradation defect of *afg3 rca1*, we measured the stability of radiolabeled proteins synthesized by isolated mitochondria from wild-type cells, from the *afg3 rca1* double mutant, or from the double mutant overproducing wild-type or proteolytically inactive Lon (Fig. 2C). When Afg3p and Rca1p were present, the half-life of mitochondrially synthesized polypeptides was ~10 min, whereas in the absence of these proteases, most of the mitochondrially synthesized proteins were stable. Overproduction of wild-type Lon or the Lon S1040A mutant failed to restore proteolysis in *afg3 rca1* mitochondria.

Because overproduction of Lon did not rescue the proteolysis defect of *afg3 rca1*, we examined whether it suppressed the defect

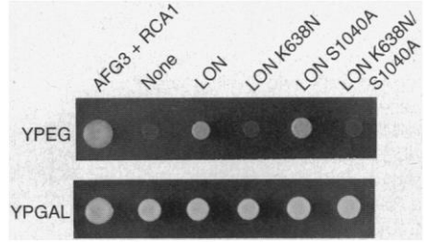

Fig. 1. The growth defect of *afg3 rca1* is suppressed by overproduction of wild-type or proteolytically inactive Lon. The *afg3 rca1* double mutant was transformed with two centromere-based plasmids carrying the *AFG3* and *RCA1* genes (AFG3 + RCA1) (*16*). Alternatively, *afg3 rca1* was transformed with the "empty" 2μ-based plasmid, YEplac181, or with this plasmid carrying either the wild-type *LON* gene (LON) or one of three different mutant *LON* genes (LON K638N, LON S1040A, or LON K638N/S1040A) (*16*). Each plasmid-encoded gene was under the control of its authentic promoter. Transformants were grown in selective liquid medium with 1% galactose for 16 hours at 30°C, suspended to an optical density of 1 at 600 nm, spotted onto agar plates containing rich medium with either 3% ethanol and 3% glycerol (YPEG) or 1% galactose (YPGAL), and grown at 30°C for 6 or 4 days, respectively.

in protein complex assembly. Assembly of Cox in *afg3* cells is partially defective, even though the levels and processing of mitochondrially and cytoplasmically synthesized subunits are normal (*10*). To assess the assembly of Cox, we exploited the fact that subunits of the fully assembled complex are trypsin-resistant, whereas the unassembled subunits are trypsin-sensitive (*21*). We monitored the trypsin resistance of Cox subunits IV, V, and VI in detergent-solubilized mitochondria; our analysis focused on the effect of the proteolytically inactive Lon because of its stronger suppression. In phenotypically wild-type cells (ΔΔ + AFG3/RCA1), 59% of subunit IV, 52% of subunit V, and 35% of subunit VI were resistant to trypsin at 100 μg/ml (Fig. 3); for *afg3 rca1* (ΔΔ), the corresponding values were only 20%, 0%, and 8.5%, respectively.

Overproduction of Lon S1040A (ΔΔ + LON S1040A) increased the resistance levels of subunits IV, V, and VI to 47%, 51%, and 21%, respectively.

We examined whether overproduction of Lon S1040A could also promote the assembly of the F_1F_0 ATPase complex in *afg3 rca1* cells. Mitochondrial protein complexes isolated from phenotypically wild-type cells (ΔΔ + AFG3/RCA1), from the *afg3 rca1* double mutant (ΔΔ), or from the double mutant suppressed by overproduced Lon S1040A (ΔΔ + LON S1040A) were resolved by blue native gel electrophoresis (*22*). Immunoblotting for the α, β, and γ subunits of F_1 indicated two discrete bands (Fig. 4A). The band migrating more slowly on the native gel contained both F_1 and F_0 subunits and thus represented the fully assembled F_1F_0 ATPase complex, whereas the faster migrating band contained only F_1 (Fig. 4B). Quantitation of the two bands indicated

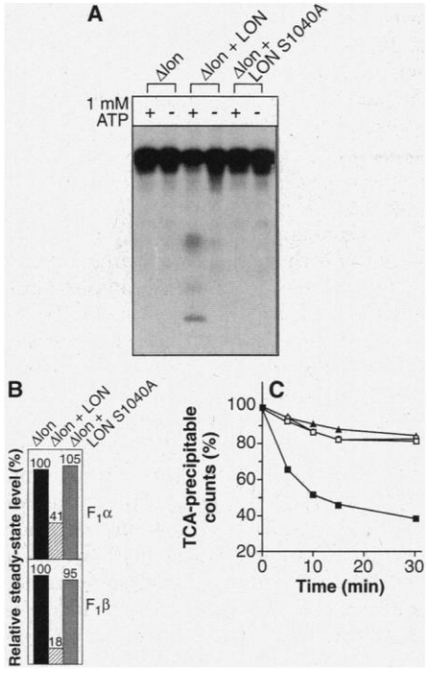

Fig. 2. The proteolysis defect in *afg3 rca1* is not suppressed by overproduction of wild-type or proteolytically inactive Lon (*27*). (**A**) Lon S1040A is proteolytically inactive in vitro. Yeast cells in which the *LON* gene had been replaced by the bacterial kanamycin gene were transformed with the "empty" plasmid pSEYc68 (Δlon) or with the plasmid carrying either the wild-type (Δlon + LON) or the proteolytically defective (Δlon + LON S1040A) *LON* gene driven by the *GAL1* promoter. In this experiment, both *LON* genes encoded a protein with a COOH-terminal hexahistidine tag. Proteins isolated on Ni^{2+}-agarose were assayed for the ability to degrade ^{125}I-labeled casein in the absence or presence of 1 mM ATP, followed by SDS-PAGE and autoradiography. (**B**) Lon S1040A is proteolytically inactive in vivo. Steady-state levels of the α and β subunits of the F_1 ATPase were analyzed by SDS-PAGE and immunoblotting of total proteins from strains in which the *LON* gene had been replaced by the bacterial kanamycin gene; the cells carried either an "empty" plasmid pSEYc68 (Δlon) or a plasmid encoding the wild-type (Δlon + LON) or the proteolytically defective (Δlon + LON S1040A) Lon protein. Cells were grown on selective galactose-containing medium that also contained 0.2% dextrose. Relative steady-state levels of $F_1\alpha$ and $F_1\beta$ were determined from densitometric analyses of autoradiographs normalized to the level of the outer membrane protein porin, and the amount of $F_1\alpha$ or $F_1\beta$ in the Δlon cells was taken as 100%. (**C**) The *afg3 rca1* proteolysis defect is not complemented by the wild-type or proteolytically inactive Lon. The *afg3 rca1* double mutant was transformed with two centromere-based plasmids carrying the corresponding wild-type genes (ΔΔ + AFG3/RCA1; solid squares) (*16*). Alternatively, *afg3 rca1* was transformed with the "empty" centromere-based plasmid pSEYc68 (ΔΔ; open squares) or with a plasmid carrying either the wild-type *LON* (ΔΔ + LON; circles) or proteolytically inactive *LON* (ΔΔ + LON S1040A; triangles) under the control of the *GAL1* promoter (*17*). Polypeptides synthesized in isolated mitochondria were pulse-labeled with [^{35}S]methionine, chased with cold methionine, and assayed for TCA-precipitable radioactivity for the time periods indicated. TCA-insoluble radioactivity at the beginning of the chase (t = 0) was taken as 100%.

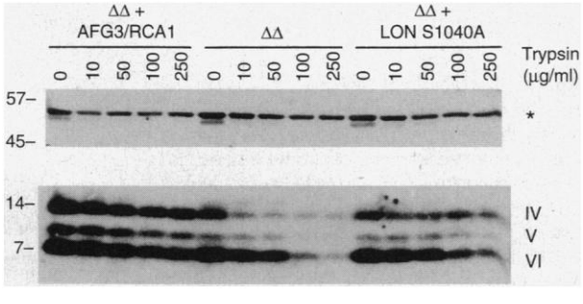

Fig. 3. Defective assembly of Cox in *afg3 rca1* is suppressed by overproduction of proteolytically inactive Lon (*28*). Mitochondria were isolated from the *afg3 rca1* double mutant transformed with two centromere-based plasmids carrying the *AFG3* and *RCA1* genes (ΔΔ + AFG3/RCA1), with an "empty" plasmid pSEYc68 (ΔΔ), or with a plasmid carrying the LON S1040A gene under the control of the *GAL1* promoter (ΔΔ + LON S1040A). Samples were treated with trypsin (0, 10, 50, 100, or 250 μg/ml) and were analyzed by SDS-PAGE followed by immunoblotting with antisera to Cox subunits IV, V, and VI. A nonspecific trypsin-resistant protein (*) recognized by the antisera indicates that equal amounts of protein have been loaded onto each lane. Relative molecular masses are indicated at the left (in kilodaltons).

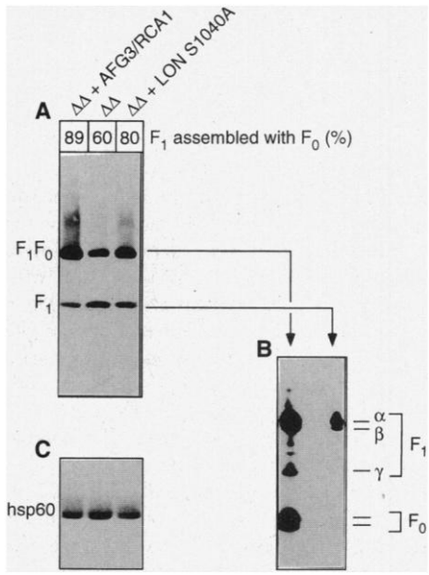

Fig. 4. Defective assembly of the F_1F_0 ATPase in *afg3 rca1* is suppressed by overproduction of proteolytically inactive Lon (*29*). (**A** and **C**) Mitochondrial proteins isolated from the *afg3 rca1* double mutant transformed with plasmids carrying *AFG3* and *RCA1* (ΔΔ + AFG3/RCA1), with an "empty" plasmid pSEYc68 (ΔΔ), or with a plasmid encoding the proteolytically inactive Lon (ΔΔ + LON S1040A) were separated by blue native gel electrophoresis. The separated proteins were analyzed by immunoblotting with antisera to the α, β, and γ subunits of F_1 ATPase and to mitochondrial hsp60. In (A), the percentage of F_1 assembled with F_0 [that is, $(F_1F_0 \times 100\%)/(F_1 + F_1F_0)$] was calculated from densitometric analyses of autoradiographs. (**B**) The upper and lower bands recognized by the F_1 antiserum were excised from the native gel and further analyzed by SDS-PAGE and immunoblotting with antisera to the F_1 and F_0 moieties of the ATPase.

that in wild-type cells, 89% of F_1 was assembled with F_0. In the *afg3 rca1* double mutant, only 60% of F_1 was assembled, whereas in the double mutant suppressed by overproduction of Lon S1040A, 80% of F_1 was assembled with F_0. Moreover, *afg3 rca1* contained only 56% of fully assembled F_1F_0 relative to the wild type, whereas the suppressed cells contained 80%. In contrast, the levels of hsp60 were essentially the same (Fig. 4C).

Overproduction of the wild-type or proteolytically inactive Lon might indirectly suppress the growth and assembly defects of *afg3 rca1* by inducing the overproduction of mitochondrial chaperones such as cpn10, hsp60, hsp70, or hsp78. However, the amounts of these chaperones were virtually identical in the *afg3 rca1* mutant and in *afg3 rca1* overproducing Lon or Lon S1040A (*23*) (Fig. 4C). Furthermore, overproduction of these chaperones (*24*) failed to restore growth of *afg3 rca1* on ethanol and glycerol (*23*).

We have thus demonstrated an unexpected functional overlap between Afg3p, Rca1p, and Lon in protein complex assembly. This common function is separable from the proteolytic activities of these proteins because the protease-defective Lon suppresses the growth and assembly defects of the *afg3 rca1* double mutant even better than does wild-type Lon. In addition, it is known that the defect in respiration-dependent growth of an *afg3* mutant is complemented by an Afg3p variant with a point mutation within the HEXXH motif, which is essential for its protease activity (*25*). Thus, the requirement for Afg3p in respiration-dependent growth is independent of its proteolytic activity and most likely depends on its ability to facilitate the assembly of respiratory complexes at the inner mitochondrial membrane.

In contrast, suppression of the growth and assembly defects of the *afg3 rca1* double mutant by Lon was abolished by a mutation within the ATP-binding motif of Lon. The sequences of this bipartite motif are GPPGVGKT and DEID for Lon and GPPGTGKT and DEID for Afg3p and Rca1p (*7*). Apart from this, Lon shares no sequence similarity with Afg3p or Rca1p; the common function of these three proteins is thus probably mediated by the regions that contain the ATP-binding site. This region may function as an ATP-regulated chaperone by binding nonnative proteins, thereby facilitating protein assembly or degradation (*26*). The chaperone-like function of Lon is apparently preserved upon inactivation of its proteolytic site. Recognition of unassembled subunits by the chaperone domain of overproduced wild-type or proteolytically inactive Lon may promote the assembly of F_1F_0 ATPase and Cox, which is normally mediated by Afg3p and Rca1p. Once assembled, the subunits may no longer be able to bind to the chaperone region. Whether a protein is degraded or assembled is perhaps determined by the affinity of this region for a protein substrate. Taken together, our results suggest a potential dual function of Lon, in mediating both the assembly of protein complexes, and the disposal of those substrates that do not assemble correctly.

REFERENCES AND NOTES

1. R. Tauer *et al.*, *FEBS Lett.* **353**, 197 (1994); E. Guélin, M. Rep, L. A. Grivell, *Yeast* **10**, 1389 (1994).
2. R. Schnall *et al.*, *Yeast* **10**, 1141 (1994).
3. A. Tzagaloff, J. Yue, J. Jang, M.-F. Paul, *J. Biol. Chem.* **269**, 26144 (1994).
4. T. Tomoyasu *et al.*, *J. Bacteriol.* **175**, 1344 (1993).
5. F. Confalonieri and M. Duguet, *Bioessays* **17**, 639 (1995).
6. J. E. Walker, M. Saraste, M. J. Runswick, N. Gay, *EMBO J.* **1**, 945 (1982).
7. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, uncharged amino acid.
8. T. Rawlings and A. J. Barrett, *Methods Enzymol.* **248**, 183 (1995).
9. A. Pajic, R. Tauer, H. Feldmann, W. Neupert, T. Langer, *FEBS Lett.* **353**, 201 (1994).
10. M.-F. Paul and A. Tzagaloff, *ibid.* **373**, 66 (1995).
11. M. Rep, J. Nooy, E. Guélin, L. A. Grivell, *Curr. Genet.* **30**, 206 (1996).
12. N. Altamura, N. Capitanio, N. Bonnefoy, S. Papa, G. Dujardin, *FEBS Lett.* **382**, 111 (1996); N. Bonnefoy, F. Chalvet, P. Hamel, P. P. Slonimski, G. Dujardin, *J. Mol. Biol.* **239**, 201 (1994).
13. M. Rep and L. A. Grivell, *FEBS Lett.* **388**, 185 (1996).
14. C. K. Suzuki, K. Suda, N. Wang, G. Schatz, *Science* **264**, 273 (1994); *ibid.*, p. 891.
15. L. Van Dyck, D. A. Pearce, F. Sherman, *J. Biol. Chem.* **269**, 238 (1994).
16. The *afg3 rca1* double mutant, formally called WDAR2(167), has been described (*11*). Conversion of *afg3 rca1* mutants to phenotypically wild-type cells was done by cotransformation of plasmids YCplac33 and YCplac111 (carrying the *AFG3* and *RCA1* genes, respectively) under the control of their authentic promoters.
17. The wild-type and mutant *LON* genes were overexpressed either on the 2μ plasmid YEplac181, under the control of the authentic *LON* promoter, or on the centromere-based plasmid pSEYc68, under the control of the *GAL1* promoter. The wild-type *LON* gene was cloned into the Bam HI and Not I sites of pSEYc68. *LON* gene mutants were produced by the polymerase chain reaction (PCR). Primer pairs used for creating LON K638N were 5′-gttaagagctctagcgatagatttacctatggacgtAttaccaacacctgggggtcctacg3′ and 5′-ggatactgaaacgaccgt-3′. The amplified product was digested with Afl II and Sac I; this fragment was used to replace the corresponding region in the wild-type *LON* gene. The mutant LON S1040A was produced in a two-stage PCR reaction; first-stage primers were 5′-gtgacaccAgcagCtggaccatct-3′ and 5′-caggatatgttgctgaa-3′. The first primer bears a t → A silent mutation removing a Pst I site and an a → C substitution replacing serine with alanine. The amplified product was used as a megaprimer in a second-stage reaction with primer 5′-cgtattcacatccttg-3′. The amplified product was digested with Xba I and Sna BI and cloned into the corresponding region of the wild-type *LON* gene. The 2μ constructs were made by cloning the wild-type *LON* gene into the Sal I site of YEplac181, and then cloning the Nco I and Bgl II fragment of the mutated genes into the corresponding sites of the wild-type gene.
18. Immunoblot analysis of total cell extracts confirmed that the wild-type and mutant Lon proteins were stably overproduced at identical levels in the *afg3 rca1* cells (M. Rep and C. K. Suzuki, data not shown).
19. A. Y. Amerik *et al.*, *FEBS Lett.* **287**, 211 (1991).
20. M. Saraste, P. R. Sibbald, A. Wittinghofer, *Trends Biochem. Sci.* **15**, 430 (1990).
21. T. L. Mason, R. O. Poyton, D. C. Wharton, G. Schatz, *J. Biol. Chem.* **248**, 1346 (1973).
22. H. Schägger and G. von Jagow, *Anal. Biochem.* **199**, 223 (1991).
23. Total cellular proteins were extracted from *afg3 rca1* cells that had been transformed with an empty plasmid (pSEYc68) or with a plasmid carrying the wild-type LON or mutant LON S1040A gene and grown on galactose-containing selective medium. Immunoblot analysis indicated that the levels of cpn10, hsp60, mitochondrial hsp70, and hsp78 were comparable. In addition, transformation of the *afg3 rca1* double mutant with plasmids resulting in the overproduction of these mitochondrial chaperones did not restore growth of the *afg3 rca1* double mutant on ethanol and glycerol medium (M. Rep, J. M. van Dijl, C.K. Suzuki, data not shown).
24. E. A. Craig *et al.*, *Mol. Cell. Biol.* **9**, 3000 (1989); S. A. Leonhardt, K. Fearson, P. N. Danese, T. L. Mason, *ibid.* **13**, 6304 (1993); D. S. Reading, R. L. Hallberg, A. M. Meyers, *Nature* **337**, 655 (1989); S. Rospert, T. Junne, B. S. Glick, G. Schatz, *FEBS Lett.* **335**, 358 (1993).
25. E. Guélin, M. Rep, L. A. Grivell, *FEBS Lett.* **381**, 42 (1996).
26. I. Levchenko, L. Luo, T. A. Baker, *Genes Dev.* **9**, 2399 (1995); S. Wickner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12218 (1994).
27. Cells were precultured to mid-log phase in selective medium (2% galactose and 0.67% yeast nitrogen base), diluted 10-fold into rich medium (2% galactose, 2% peptone, and 1% yeast extract), and grown to mid-log phase. Mitochondria were isolated as described [B. S. Glick and L. Pon, *Methods Enzymol.* **260**, 213 (1995)], except that the cells were homogenized in 20 mM tris-HCl (pH 7.5), 0.6 M sorbitol, aprotinin (100 μg/ml), 1 mM *o*-phenanthroline, 100 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and 100 μM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). Mitochondria were suspended at 5 mg/ml in solubilization buffer [20 mM tris-Cl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 100 μM TLCK, 100 μM TPCK, aprotinin (100 μg/ml), and Lubrol WX (1.6 mg per milligram of protein)]. Insoluble material was removed by centrifugation (200,000*g*, 5 min, 4°C), and the supernatant was incubated with 50 μl of Ni^{2+}-agarose for 90 min at 4°C with gentle agitation. The beads were washed five times with solubilization buffer containing 20 mM imidazole and were divided into two aliquots, and excess buffer was removed. Each aliquot was incubated for 40 min in a final volume of 40 μl at 37°C, in the presence or absence of 1 mM ATP with 25 mM tris-Cl (pH 8.0), 12.5 mM $MgCl_2$, and ^{125}I-labeled casein (150,000 cpm, or ~15,000 cpm per microgram of casein). Reducing sample buffer (50 μl, 2×) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to terminate the reaction, and each sample was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (15%) and autoradiography. To assess the stability of proteins synthesized within mitochondria, we precultured the transformants in selective medium and inoculated them into a large-scale culture, as described above. Mitochondria were isolated and labeled for 20 min at 25°C with [^{35}S]methionine essentially as described [T. Langer, A. Pajic, I. Wagner, W. Neupert, *Methods Enzymol.* **260**, 495 (1995)]. Labeling was terminated with 20 mM unlabeled methionine and 100 μM puromycin; the mitochondria were washed three times with 0.6 M mannitol, 2 mM EGTA, and 10 mM tris-maleate (pH 6.8), incubated at 37°C, and assayed for trichloroacetic acid (TCA)–precipitable radioactivity at the indicated times.

28. Mitochondria were isolated from transformants precultured in galactose-containing selective medium and then inoculated into selective medium containing 0.3% yeast extract. Mitochondria (3 mg of protein) were suspended in 150 μl of 50 mM Na_2HPO_4, 0.9% NaCl, and 1 mM EDTA and were solubilized by addition of 100 μl of 10% Na-cholate (pH 7.8) for 15 min on ice. After centrifugation at 100,000*g* for 15 min at 4°C, the supernatant was divided into 40-μl aliquots and incubated for 30 min on ice with trypsin (0, 5, 10, 100, or 250 μg/ml). After addition of PMSF to a final concentration of 1.2 mM and incubation for 10 min, 0.45 ml of reducing sample buffer (2×) was added to each aliquot. A 60-μl portion of each sample was analyzed by SDS-PAGE followed by immunoblotting with antisera to Cox subunits. The immunoblot was developed by ^{125}I-labeled protein A and autoradiography.
29. For assessment of the assembly state of the F_1F_0 ATPase, mitochondria isolated from the various transformants (400 μg) were suspended in 40 μl of 0.75 M 6-aminocaproic acid, 50 mM bistris-HCl (pH 7.0), and 1 mM PMSF and were solubilized by addition of 7.5 μl of 10% lauryl maltoside. After centrifugation at 100,000*g* for 15 min at 4°C, the supernatant was mixed with 2.5 μl of 5% Serva blue G and electrophoresed on a 5 to 12% nondenaturing polyacrylamide gel (*22*). After electrophoresis, the gel was incubated for 30 min with 50 mM bistris-HCl and 15 mM tricine-HCl (pH 7.0). The separated proteins were analyzed by immunoblotting with antisera to the α, β, and γ subunits of F_1 ATPase and to mitochondrial hsp60. For quantitation, different amounts of protein were loaded to ensure that measurements were within the linear range; these different samples were prepared from 800, 400, and 200 μg of mitochondrial protein.
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Requirement for Invariant Chain in B Cell Maturation and Function

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Previously the role of invariant chain (Ii) had been described only as a chaperone that facilitates folding and transport of major histocompatability complex class II molecules; here it is shown that Ii is required for B cell development. B cells from mice lacking Ii were found to have a low response to T-independent type II antigen and could not proliferate after the mice were injected with antigen. Study of cell surface markers revealed a developmental arrest that prevented immature virgin B cells from becoming mature B cells in the periphery. This block was independent of major histocompatability complex class II expression and was an intrinsic feature of B cells that correlated with the amount of Ii. Thus, Ii participates by an unknown mechanism in B cell maturation.

Major histocompatability complex (MHC) class II molecules associate with trimers of Ii during biosynthesis. Ii facilitates folding of class II molecules, interferes with their association with peptides, and is involved in MHC class II transport (*1*). Furthermore, elimination of the Ii gene by gene targeting greatly diminishes the ability of antigen-presenting cells (APCs) to present exogenous protein antigen in a class II–restricted fashion and impairs the maturation of $CD4^+$ T cells in the thymus (*2–4*). The assembly, transport, and function of MHC class II have been studied in detail in mice lacking Ii (*2–4*). There has not, however, been a rigorous examination of the functional capability of B cells. We therefore analyzed the function of B cells lacking Ii.

To examine the function of B cells lacking Ii (Ii^-), we measured B cell response both to the type II thymic-independent (TI) antigen NP-Ficoll and to NP-CGG, a thymic-dependent (TD) antigen (Fig. 1). Both TD and TI responses were markedly reduced in the Ii^- mice. The Ii^- mice have reduced numbers of $CD4^+$ T cells, which predicts that these mice should have weak responses to TD antigen. However, unlike the response in class II–deficient mice (*5, 6*), 6 days after immunization, concentrations of immunoglobulin M (IgM) to NP were low, suggesting that the primary response of B cells was also impaired. This observation is consistent with the defective primary antibody response by the Ii^- mice after keyhole limpet hemocyanin (KLH) injection (*2*). In response to NP-Ficoll, B cells lacking Ii produced little IgM both 6 and 14 days after injection. Thus, the B cells in Ii^- mice were unable to respond normally to TI antigen (Fig. 1).

Equivalent numbers of $B220^+$ B cells were found in the periphery of the control mice, Ii^- mice, and two lines of transgenic Ii mice that express low amounts of one of the two isoforms of Ii, p31 and p41 (designated Ii^{p31lo} and Ii^{p41lo}, respectively) (*7, 8*). To determine the ability of these B cells to respond to antigen in vivo after stimulation, we immunized mice with KLH and examined draining lymph node B cells 9 days later. In the draining lymph nodes of control mice, the $B220^+$ B cell population had proliferated and increased to 54.3 ± 6.8% of the total cells from 13.75%. In the absence of Ii or in the presence of low amounts of p31 or p41 Ii, however, the B cell population expanded to only 26.6 ± 3.2% (*9, 10*). Because in both Ii^{p31lo} and Ii^{p41lo} mice $CD4^+$ T cells are present in normal amounts (*7*), this low proliferation cannot be attributed to the $CD4^+$ T cell deficiency. Thus, the low number of B cells found after immunization with protein antigen could be explained by a defect in the B cell response or by the rapid death of these cells. B cells from Ii^- mice proliferated as well in vitro as did wild-type cells in the presence of lipopolysaccharide (LPS) (*11*).

B cell development occurs independently of MHC class II expression (*5, 6*). To analyze B cell maturation in the absence of Ii, we compared spleen cells from control and Ii knockout mice (Ii^-) using a panel of antibodies to B cell markers. Unlike B cells from control mice, the Ii^- B cells expressed lower

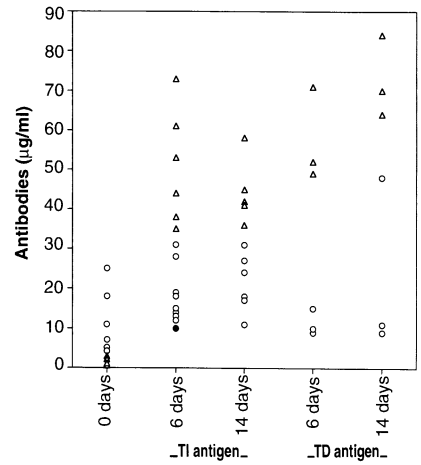

Fig. 1. Immunoglobulin M response to TD and TI antigens. Ii^- (circles) or wild-type littermates (triangles) were challenged with the type II-TI antigen NP_{90}-Ficoll or with the TD antigen NP_{16}-CGG. At the indicated times after injection, blood was drawn and IgM titers were quantitated by enzyme-linked immunosorbent assay (*21*). The concentration of antibodies at time zero in the absence of immunization probably represents basal concentrations of low-affinity IgM, although it is not clear why this amount is higher in the Ii^- mice than in the control mice. Mean titers (micrograms per milliliter) were as follows. Day 0: control, 1.415 ± 0.96 (n = 6); Ii^-, 10.7 ± 7.9 (n = 7). Day 6: control, 50.6 ± 14.6 (n = 6); Ii^-, 17.2 ± 7.06 (n = 10). Day 14: control, 43.8 ± 7.5 (n = 6); Ii^-, 19.85 ± 7.75 (n = 7). The symbol that appears solid represents two Ii^- mice with identical antibody concentrations.

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