p32 Protein, a Splicing Factor 2-associated Protein, Is Localized in Mitochondrial Matrix and Is Functionally Important in Maintaining Oxidative Phosphorylation*

(Received for publication, June 11, 1997)

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Human p32, originally cloned as a splicing factor 2-associated protein, has been reported to interact with a variety of molecules including human immunodeficiency virus Tat and complement 1q (C1q). p32 protein is supposed to be in the nucleus and on the plasma membrane for the association with human immunodeficiency virus Tat and C1q respectively. None of the interactions, however, is proven to have a physiological role. To investigate the physiological function of p32, we determined the intracellular localization of p32. The fractionation of cells, fluorescent immunocytochemistry, and electron microscopic immunostaining show that p32 is exclusively localized in the mitochondrial matrix. We cloned a Saccharomyces cerevisiae homologue of human p32 gene, referred to yeast p30 gene. The yeast p30 protein is also localized in the mitochondrial matrix. The disruption of the p30 gene caused the growth retardation of yeast cells in a glycerol medium but not in a glucose medium, i.e. the impairment of the mitochondrial ATP synthesis. The growth impairment was restored by the introduction of the human p32 cDNA, indicating that p30 is a functional yeast counterpart of human p32. Taken together, both p32 and p30 reside in mitochondrial matrix and play an important role in maintaining mitochondrial oxidative phosphorylation.

Human p32 has been cloned as a splicing factor 2-associated protein from human HeLa cells (1). This protein has been reported to interact with a variety of substances. The p32 protein has been shown to interact with human immunodeficiency virus (HIV) Tat protein (2–4) and Rev protein (5, 6) by using in vitro binding studies and two-hybrid analyses in yeast cells. In the cell transfected with the HIV Tat cDNA, the promoter-bound Tat can recruit a p32/VP16 fusion protein to the promoter (3). The transient expression of the murine homologue of p32, YL2, potentiates the function of Rev up to 4-fold (5). Hence, it is presumed that p32 protein might be a co-factor of HIV Tat and Rev proteins. The transcription factor IIB (7) and lamin B receptor (8, 9) have also been reported to interact with the p32 protein in vitro.

In addition to these proteins, p32 can interact with globular heads of C1q (gC1q). Because the recombinant p32 protein inhibits C1q hemolytic activity (10, 11), p32 is assumed to be a receptor for gC1q. Furthermore, the binding of p32 to H-kininogen (12), to hyaluronic acid (13), and to vitronectin (11) has been reported.

Irrespective of the binding of p32 to many substances, the functional aspects of these associations are not appropriately defined. For example, p32 is neither cross-linked to RNA nor effective to the splice site selection (1), casting a question about the contribution to splicesome. p32 must be localized in the nucleus to functionally associate with SF2/ASF, HIV Tat, Rev, transcription factor IIB, and lamin B receptor. Although p32 is proposed to be in the nucleus (8), it is not unambiguous because the crude nuclear fraction, a 1,000 x g pellet, is usually contaminated with other fractions of cells (15). To interact with gC1q, H-kininogen, hyaluronic acid, and vitronectin, p32 must exist at the extracellular side of plasma membrane. There is evidence for the presence of p32 in the membrane fraction (13). The reported membrane fraction, which is prepared by 30,000 x g centrifugation of post-nuclear supernatant (13), would contain many organelles including mitochondria which are pelletted by 7,000 x g centrifugation. More recently, most of p32 has been suggested to be localized in cytoplasm but neither in nucleus nor on plasma membrane (16).

In addition to uncertainty of p32 localization, the possibility is raised that p32, which is a highly acidic protein with pI 4.2, nonspecifically interacts with the basic region of nuclear proteins (4).

Thus, despite the many studies on p32, its physiological role remains to be clarified. To elucidate the function of p32, we cloned yeast p30 gene, a yeast counterpart of human p32, and precisely examined the intracellular localization of human p32 and yeast p30 proteins. Here we show that both p32 and p30 proteins are exclusively localized in the mitochondrial matrix and that they are functionally important in maintaining mitochondrial oxidative phosphorylation.

EXPERIMENTAL PROCEDURES

Fractionation of U937 Cells—U937 cells were cultured in RPMI 1640 media (Life Technologies, Inc.) containing 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO₂ humidified incubator. After the

* This work was supported in part by Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby

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growth reached 60–70% confluence, the cells were harvested and ho-
mogenized in the homogenizing buffer (10 mM Tris-HCl, pH 7.4, 0.25 m
sucrose, and 1 mM EDTA). The subcellular fractions were obtained as described (17). Briefly, the crude mitochondrial fraction (7,000 × g pellet of 700 × g supernatant) was layered on the discontinuous sucrose gradient, consisting of successive layers of 1.6, 1.0, 0.5 m sucrose against the bottom and centrifuged at 100,000 × g. The band between 1.6 and 1.0 m sucrose was collected for the mitochondrial fraction. The crude nucleic acid (700 × g pellet) was loaded on the top of discontinuous sucrose gradient made by successive layering of 4.5 ml of 2.0 and 1.6 m sucrose containing 1 mM MgCl2 and centrifuged at 100,000 × g for 60 min. The pellet at the bottom was collected and used for nuclear fraction.

Fractionation of Mitochondria—The mitochondrial fraction was further separated (17). Three mg of the mitochondrial fraction in 0.5 ml of buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose) was sonicated at output 5 for 10 s with an Ultrasonic processor (Tokyorika, Tokyo, Japan) 5 times and centrifuged at 100,000 × g. The resulting pellets and supernatant were used as membrane and soluble fractions, respectively. To disrupt outer membrane, the mitochondrial fraction was suspended in a hypotonic buffer (10 mM HEPES, pH 7.4, 1 mM EDTA) and centrifuged at 14,000 × g for 10 min. The pellets and supernatant were used as mitoplasts and intermembranous fractions, respectively.

Vectors for Construction for p32 cDNA—Human cDNA library (Human HeLa S3 MATCHMAKER cDNA Library, CLONTECH Laboratories, Inc., Palo Alto, CA) was used for amplification of human p32 cDNA.

The DNA fragment corresponding to the mature form of p32-(74–242) was amplified by PCR using gene-specific primers H74 (5′-GAAT-
TCTTGGCGAGTGTCTCT-3′) containing EcoRI site at its 5′ end and H282TG (5′-GGATCCGGTCTACTGCTTGAGAC-3′) containing BamHI site at its 5′ end and stop codon of p32. The PCR product was subcloned into the vector pCR®II (Invitrogen BV, De Schelv, NV, Leek, The Netherlands) to generate pCRHm and then the sequence of the insert was examined to be correct.

The EcoRI-BamHI fragment from pCRHm was inserted into the vector pBLS15b (Strategene Ltd., Innovation Center, Cambridge). Then the vector was digested by XhoI and BamHI. The XhoI-BamHI fragment was inserted into XhoI-BamHI sites of the vector pET15b (Novagen, Madison, WI) to generate the expression vector pETHm for producing a mature form of human p32 with His-tag at its N terminus in Escherichia coli.

Vectors for Transfection—The vectors for expressing hemagglutinin (HA)-tagged p32 protein in PLC cells were constructed as follows. First, a 1.6-kb fragment containing cDNA for HA-Tag (63 amino acids) was made. This fragment (XhoI site at its 5′ end and BamHI site at its 3′ end) was inserted into the vector pETHm, to generate pETHm-HA. The XhoI-BamHI fragment of human p32 was amplified by PCR from the cDNA library with gene-specific primers H1 (5′-GAATTTCTCTCGGATGCTTCTCT-3′) containing EcoRI site at its 5′ end and H282TG (5′-GGATCCGGTCTACTGCTTGAGAC-3′) containing BamHI site at its 5′ end. To generate a DNA fragment coding a mature form of p32-(74–242) without stop codon, a PCR reaction was performed using primers H282 and H37ATG (5′-GAATTCATCAGCTGGACGCGAGCAGCAGCAGCTGG-3′) encoding the 12 amino acids of influenza EA epitope plus six histidine repeat was annealed with its antisense oligonucleotide. The BamHI-XhoI fragment of this DNA was inserted into BamHI-XhoI sites of the vector pBLS15b (Novagen, Madison, WI) to generate pBH1.

The EcoRI-BamHI fragment of human p32 was amplified by PCR from the cDNA library with gene-specific primers H1 (5′-GAATTTCTCTCGGATGCTTCTCT-3′) containing EcoRI site at its 5′ end and H282TG (5′-GGATCCGGTCTACTGCTTGAGAC-3′) containing BamHI site at its 5′ end. To generate a DNA fragment coding a mature form of p32-(74–242) without stop codon, a PCR reaction was performed using primers H282 and H37ATG (5′-GAATTCATCAGCTGGACGCGAGCAGCAGCAGCTGG-3′) encoding the 12 amino acids of influenza EA epitope plus six histidine repeat was annealed with its antisense oligonucleotide. The BamHI-XhoI fragment of this DNA was inserted into BamHI-XhoI sites of the vector pBLS15b (Novagen, Madison, WI) to generate pBH1.

Vectors for Yeast Transformation—A PCR reaction was performed with the primer H1 and H282TG, and the PCR product was subcloned into pCR®II, the sequence was compatible with those previously reported (18). The EcoRI fragment containing p32 full-length cDNA was inserted into pGAP (19). Yeast protein expression was performed in the yeast strain YPH500 (MATa, ura 3-52, lys 2-801, ade 2-101, trp 1-63, his 3-300, leu 2-1) by Ito et al. (26). Yeast protein expression was performed in the yeast strain YPH500 (MATa, ura 3-52, lys 2-801, ade 2-101, trp 1-63, his 3-300, leu 2-1) by Ito et al. (26). Yeast cells were grown in the medium consisting of 1% yeast extract, 2% Bacto-peptone, and 2% glucose (YPD) or 3% glycerol (YPG). Solid medium consisted of 2% Bacto-agar, 1% yeast extract, 2% Bacto-peptone, and 2% glucose (YPDagar) or 3% glycerol (YPGagar). Yeast transformation was performed by the lithium chloride procedure as described by Ito et al. (24). Mating the haploid strain was performed as described by Kaiser et al. (25). Total lysate of yeast cells for SDS-PAGE was prepared as described by Yamazaki et al. (26). Data base sequence comparison was performed using FASTA and BLAST program as described (27)

Vectors for Yeast p30—The yeast p30 gene encoding 1–266 amino acids was amplified with a primer UY1 (5′-AAGCTTAAAAACAAACT-AATGGCTTCTC-3′) containing HindIII site at its 5′ end and a primer LY266 (5′-GGATCCAGTGGAAGAATTCTTCTCA-3′) containing BamHI site at its 5′ end. The PCR was performed on genomic DNA.
from yeast strain YPH499. The PCR product was subcloned into the vector pcR<sup>®</sup>II to generate pCRY1. Two independent clones in the sequences completely matched those deposited in GenBank were used for yeast p30 cDNA. A vector of yeast p30 (pGAPY1) for the protein expression in yeast cells was constructed by inserting EcoRI fragment from pCRY1 into EcoRI site of pGAP.

To construct a cDNA for a GST–yeast p30-(114–266) fusion protein, a DNA fragment containing 114–266 amino acids of p30 was amplified with a gene-specific primer MY (5′-GAATTCATGGACGTAGCTCA-GATTGCTAAT-3′) containing EcoRI site at its 5′-end and the primer LY266. The PCR product was subcloned into pCR<sup>®</sup>II to generate pCRY114. EcoRI-BamHI fragment from pCRY114 was inserted into pGEX-4TK vector (Pharmacia). The resulting expression vector pGEXY114 was used for producing a GST–yeast p30-(114–266) fusion protein in E. coli.

**Disruption of p30 Gene**—Deletion of most of the p30 gene was carried out by a one-step replacement (28). A targeting vector for yeast p30 cDNA was constructed as follows. PCR was performed on genomic DNA from yeast strain YPH500 with primers TU1 (5′-GGTTGAAAGGCT-TCCAGCAC-3′) and TU2 (5′-CATATGTGATGTTATGATCTT-3′). TU1 corresponds to 24 bp upstream of initiation codon. TU2 corresponds to 24 bp upstream of initiation ATG (A = 0 bp) codon of yeast p30 open reading frame and contains NdeI site at its 5′-end. The PCR product was subcloned into pCR<sup>®</sup>II to generate pCR-U. Another PCR reaction was performed with primers TLI (5′-TGGATCTTCTTCTGT-GATATGCTCTTCGCGTGTC-3′) and TLI2 (5′-GAGCTCAGCTGCCTGTTGC-3′). TLI is complementary 261 bp upstream of the termination site and contains the adaptor of BamHI site at its 5′-end. TLI2 is complementary 258 bp downstream of termination TAA (A = 0 bp) codon of yeast p30 open reading frame and contains the adaptor of SacI site at its 5′-end. The PCR product was subcloned into pCR<sup>®</sup>II to generate pCR-L. The 1229-bp PuI-BamHI fragment from the plasmid pGB9 (CLONTECH Laboratories, Inc., Palo Alto, CA), which contains the TRP1 yeast auxotrophic marker, was inserted into PuI and BamHI sites of pBR222 to generate pBR-T. The 1460-bp fragment containing PuI-BamHI fragment was excised from pBR-T with NdeI and BamHI. The fragment was inserted into pCR-L and pCRY114 sites of pCR-U to generate pCR-UT. The 520-bp BamHI-SacI fragment from pCR-L was inserted into BamHI and SacI sites of pCR-UT to generate pCR-UTL. The DNA for targeting was liberated from the plasmid by EcoRI-SacI digestion and used to transform the haploid strain YPH500 to generate strain YPH500 (Δp30:TRP1). To replace TRP1 by HIS3, the NdeI-SacI fragment containing TRP1 of the plasmid pCR-UTL was removed, and the resulting cohesive ends of the plasmid were filled and ligated to each other. Then a BamHI fragment from the vector pYAC3 (29) containing HIS3 was inserted into the BamHI site of the TRP1 gene-deleted plasmid to generate pCR-UHL. The EcoRI-SacI fragment from the plasmid was used to transform the haploid strain YPH499 and generate strain YPH499 (Δp30:His3). The gene disruptions of p30 were verified at the DNA level (30) and by Northern hybridization analysis (31).

**Subfractionation of S. cerevisiae**—Mitochondrial fraction was isolated from the strain YPH499 as described by McKee and Poyton (32) with minor modifications. Spheroplasts prepared by the treatment of yeast cells with zymolyase 20T (10 mg for 1 g of cells) (Seikagaku, Tokyo, Japan) were homogenized. The homogenate was centrifuged at 700×g for 10 min. The resulting supernatant was collected and centrifuged at 100,000×g for 60 min. The supernatant was used as a cytosolic fraction, and the pellet was used as a microsomal fraction. A mitochondrial fraction was prepared by osmotic shock as described by Daum et al. (33). The activity of lactate dehydrogenase was measured by the method of Bergmeyer et al. (34). One unit of the activity was defined as the absorbance change of 1.0 min. The activities of succinate-cytochrome c reductase (35), insensitive NADPH-cytochrome c reductase (36), adenylyl kinase (35), and fumarase (37) were measured as described. One unit of the activities for adenylyl kinase and fumarase was defined as the absorbance change of 1.0 min. Protein concentrations were determined by the method of Lowry et al. (38) with bovine serum albumin as a standard.
p32 Protein in Mitochondria

TABLE I
p32 in subcellular fractions of U937

| Subcellular fraction | Succinate-cytochrome c reductase | Lactate dehydrogenase | Rotenone-insensitive NADPH-cytochrome c reductase | p32 protein |
|---------------------|---------------------------------|-----------------------|-----------------------------------------------|-------------|
| Total lysate        | 32.2 ± 9.6                      | 53.1 ± 19.1           | 35.5 ± 11.9                                   | 35.0 ± 14   |
| Mitochondria        | 100a                            | 0.63 ± 0.41           | 1.37 ± 0.24                                   | 100b        |
| Cytosol             | 0.2 ± 0.5                       | 100b                  | 1.0 ± 0.6                                     | ND (<1.0)  |
| Microsomes          | 3.1 ± 1.6                       | 0.56 ± 0.34           | 100c                                          | ND (<1.0)  |

a 95.5 ± 12.5 μmol/min per mg of protein.  
b 14.0 ± 6.0 μmol/min per mg of protein.  
c 12.1 ± 5.4 μmol/min per mg of protein.  
d 1.43 ± 0.29 μg/ml/mg protein.

FIG. 2. Subcellular distribution of p32 protein in U937 cells. U937 cells were fractionated as described under “Experimental Procedures.” A, 50 ng of purified recombinant p32 protein (rp32) and 40 μg of protein of each fraction were separated on 10% SDS-PAGE. After transfer onto nitrocellulose membrane, p32 protein was detected with the affinity purified anti-p32 protein antibody. B and C, recombinant p32 (25 ng/lane) and each fraction (30 μg/lane) were analyzed as in A. mt, mitochondrial; mt soluble, mitochondrial soluble fraction; mt membrane, mitochondrial membrane fraction; intermembrane, mitochondrial intermembranous fraction; mitoplast, mitoplast fraction.

p32, the specific content of p32 in the mitochondrial fraction was 1.43 ng/mg protein (Table I). In agreement with the mitochondrial localization of p32, the relative specific content of p32 in each fraction was essentially the same as the relative specific activity of succinate-cytochrome c reductase (Table I). On the basis of recovery of succinate-cytochrome c reductase activity in the mitochondrial fraction, the amount of p32 protein in mitochondria was calculated to account for virtually all (approximately 96%) of the total amount of p32 in cells. The reason why succinate-cytochrome c reductase activity was higher than p32 content in the mitochondrial fraction (Table I) may be the contamination of the fraction with disrupted mitochondrial inner membranes lacking matrix components.

Localization of p32 Protein in the Mitochondrial Matrix—The sublocalization of p32 in mitochondria was examined. When the mitochondrial fraction was separated into membrane and soluble fractions after sonication, p32 was recovered from the soluble fraction exactly in parallel with the activity of fumarase that resides in the mitochondrial matrix (Fig. 2B) (Table II). On the basis of recovery of fumarase activity in the mitochondrial soluble fraction, the content of p32 in the soluble fraction accounted for about 96% of p32 in intact mitochondria.

To determine whether mitochondrial p32 is localized in the intermembranous space or in the matrix, we preferentially disrupted outer membranes of mitochondria by hypotonic treatment and obtained mitoplast and intermembranous fractions. p32 as well as the activities of succinate-cytochrome c reductase and fumarase was recovered from the mitoplast fraction with precisely the same degree of enrichment (Table II). p32 was negligible in the intermembranous space where the activity of adenylate kinase, a marker enzyme of the space, was enriched (Fig. 2C). On the basis of recovery of fumarase activity in the mitoplast fraction, the amount of p32 in mitoplasts accounted for about 102% p32 in intact mitochondria (Table II). These results indicate that p32 is exclusively localized in the mitochondrial matrix.

The localization of p32 in the matrix was further confirmed by electron microscopic immunostaining of mitochondria isolated from Jurkat cells by using anti-p32 antibody (Fig. 3). The signals were seen in the matrix (electron dense area). When non-immune serum was used, signals were not observed (data not shown).

p32 Protein in the Nucleus—We prepared the crude nuclear fraction and post-nuclear supernatant by centrifugation of the total cell lysate at 700 × g. The resulting crude nuclear fraction was further purified by centrifuging the fraction on the discontinuous sucrose gradient bed. p32 was recovered from the post-

FIG. 3. Electron microscopic immunostaining of mitochondria. Isolated mitochondria from Jurkat cells were stained with immune serum against p32 protein as described under “Experimental Procedures.”
nuclear supernatant (Table III). The recovered amount of p32 protein in the post-nuclear supernatant accounted for 107% of p32 content in the total lysate according to the recovery of succinate-cytochrome c reductase activity in the post-nuclear supernatant. The relative specific content of p32 in the nuclear fraction was essentially the same as the relative specific activity of succinate-cytochrome c reductase in the nuclear fraction.

### Table III

The specific content of p32 and the specific activity of succinate-cytochrome c reductase are expressed as percent of those in the mitochondrial fraction. Each value is a mean of two independent experiments. Nucleus, nuclear fraction; post-nuclear, post-nuclear supernatant fraction.

|                | Succinate cytochrome c reductase | p32 protein |
|----------------|----------------------------------|-------------|
| Total lysate   | 100a                             | 100b        |
| Nucleus        | 3.75                             | 5.5         |
| Post-nuclear   | 132.8                            | 123.7       |

a 21.62 µmol/min per mg of protein.
b 4.8 arbitrary units/mg of protein.

In the cells transfected with the full-length cDNA, the mature form of 34-kDa protein was found to be much stronger than the premature form of 38-kDa protein both with anti-HA and anti-p32 antibodies (Fig. 4A, lane 2 in left and right panels), indicating that the HA-tagged premature p32 is efficiently processed to the mature form in the cells. Only a 34-kDa protein was detected in the cells transfected with the mature form of cDNA (Fig. 4A, lane 3 in left and right panels). Endogenous p32 protein (32 kDa) was detected in all the three cell lines with anti-p32 antibody (Fig. 4A, right panel).

In the cells transfected with the full-length cDNA, HA-tagged p32 distributed granularly in the cytoplasm but not in the nucleus (Fig. 4B, left panel) as observed for endogenous p32 in Fig. 1A, indicating its mitochondrial localization. In contrast, the cytoplasm and, to a lesser extent, nucleus were homogeneously stained in the cells transfected with the mature form cDNA (Fig. 4B, right panel), irrespective of both cell lines predominantly containing the mature 34-kDa protein (Fig. 4A).

These results suggest that the N-terminal region to be processed is necessary for p32 to be imported to mitochondria.

### Intracellular Localization of Yeast p30 Protein

We have cloned a yeast gene homologous to human p32 gene, referred to yeast p30. The amino acid sequence of yeast p30 and human p32, which shows 60% similarity and 30% identity over all, is highly conserved particularly in their C termini (Fig. 5B). The human p32 and yeast p30 proteins shared well conserved structural features, i.e. (i) diffuse distribution of acidic residues with intercalated non-charged portions, (ii) small regions with positive charges at the N- and C termini, and (iii) a region with a periodic repeat of leucine residues (Fig. 5A).

When the total homogenate of wild type yeast cells was immunoblotted with anti-p30 antibody, the antibody specifically probed a protein with a molecular mass of 30.5 kDa (Fig. 6A, lane 1 in upper panel). Preimmune serum could not detect this 30.5-kDa band (data not shown). Total homogenates of wild type cells were separated into mitochondrial, cytosolic, and microsomal fractions. The contamination with mitochondrial p32 protein was determined by a Western blot procedure using antibodies specific for HA-tag (original magnification, 400 x).

### Fig. 4

**Immunofluorescence staining and immunoblot analysis of transiently transfected cells.** A, PLC cells were transfected with pCIHA1 encoding 1–282 amino acids of p32 (p32/1–282) or pCIHA74 encoding 74–282 amino acids of p32 (p32/74–282) as described under “Experimental Procedures.” The cells were suspended in PBS and sonicated. Whole cell lysates (100 µg/lane) were separated on 16% SDS-PAGE and immunoblotted using antibodies specific for HA-tag (anti-HA) and p32 (anti-p32). B, the cells were fixed and stained with antibodies specific for HA-tag distributed granularly in the cytoplasm but not in the nucleus (Fig. 4B, left panel) as observed for endogenous p32 in Fig. 1A, indicating its mitochondrial localization. In contrast, the cytoplasm and, to a lesser extent, nucleus were homogeneously stained in the cells transfected with the mature form cDNA (Fig. 4B, right panel), irrespective of both cell lines predominantly containing the mature 34-kDa protein (Fig. 4A). These results suggest that the N-terminal region to be processed is necessary for p32 to be imported to mitochondria.

### Fig. 5

**Comparison of the amino acid sequences.** A, amino acid sequences of human p32 and S. cerevisiae p30 are depicted schematically with boxed regions as indicated. B, comparison of the C-terminal amino acid of p32 from humans, YL2 protein (5) from mice, yeast p30 from S. cerevisiae, and F59A2.3 (similar to splicing factor-associated 32K chain) from Caenorhabditis elegans. Key: identity (1), high similarity (2), similarity (3).
dria was less than 1% both in the cytosolic and microsomal fractions based on the specific activity of succinate-cytochrome c reductase (Table IV). The mitochondrial heat shock protein 60 (p66) was also detected only in the mitochondrial fraction (Fig. 6A, lane 1 in lower panel), further indicating that the fractions are well separated. When these fractions were immunoblotted with anti-p30 antibody, p30 was only detected in the mitochondrial fraction (Fig. 6A, upper panel). When the mitochondrial fraction was subfractionated (Table V), p30 was detected in the soluble fraction (Fig. 6B, left panel) and the mitoplast fraction (Fig. 6B, right panel) as observed in the case of human p32. The finding indicates that yeast p30 is also localized in the mitochondrial matrix.

**Gene Targeting of p30**—To investigate the role of p30 in mitochondria, we disrupted the yeast p30 gene. When the total homogenate of the Δp30 strain (YPH499Δp30::HIS3)) was immunoblotted with anti-p30 antibody, the signal for p30 was not visible (Fig. 6C, upper panel), whereas the mitochondrial heat shock protein 60 (p66) was detected both in the wild type and in the Δp30 strain to a similar extent (Fig. 6C, lower panel). When the plasmid containing p30 cDNA was introduced into the Δp30 strain, the expression of p30 protein was restored (Fig. 6D, lane 2 in middle panel). The Δp30 strain grew normally in a medium containing glucose (YPDagar), slowly in the glycerol medium (YPGagar) at 30 °C (data not shown), and very slowly at 18 °C (Fig. 7A, Δp30), suggesting that the Δp30 strain has an abnor-
p32 Protein in Mitochondria

Subcellular fractions of yeast cells (YPH499) were prepared as described under "Experimental Procedures." The specific activities of lactate dehydrogenase, succinate-cytochrome c reductase, rotenone-insensitive NADPH-cytochrome c reductase in subcellular fractions are expressed as percent of those in cytosol, mitochondrial, and microsomal fractions, respectively. Each value is a mean of two independent experiments.

|              | Succinate-cytochrome c reductase | Lactate dehydrogenase | Rotenone-insensitive NADPH-cytochrome c reductase |
|--------------|---------------------------------|-----------------------|-----------------------------------------------|
| Total lysate | 10.4                            | 82                    | 58                                            |
| Mitochondria | 100\(^a\)                       | 3.7                   | 5.6                                           |
| Cytosol      | 1.7                             | 100\(^b\)             | 0.37                                          |
| Microsomes   | 0.33                            | 7.2                   | 100\(^c\)                                     |

\(^a\) 26.5 \mu mol/min per mg of protein.
\(^b\) 2.4 units/min per mg of protein.
\(^c\) 4.97 \mu mol/min per mg of protein.

**TABLE V**

Subfractionation of yeast mitochondria

Submitochondrial fractions of yeast (YPH499) were prepared from the mitochondrial fraction as described under "Experimental Procedures." The specific activities of succinate-cytochrome c reductase, adenylate kinase, and fumarase in each fraction are expressed as percent of those in the mitochondrial fraction. Each value is a mean of two independent experiments.

|                       | Succinate-cytochrome c reductase | Fumarase | Adenylate kinase |
|-----------------------|---------------------------------|----------|------------------|
| Mitochondria          | 100\(^a\)                       | 100\(^a\)  |                  |
| Soluble               | 1.5                             | 198.4     |                   |
| Membrane              | 128.4                           | 26.8      |                   |
| Intermembrane         | 100\(^c\)                       | 100\(^c\)  |                  |
| Mitoplast             | 5.65                            | 218.6     |                  |
|                       | 116.7                           | 15.3      |                  |

\(^a\) 17.3 \mu mol/min per mg of protein.
\(^b\) 3.7 units/min per mg of protein.
\(^c\) 2.22 units/min per mg of protein.

We examined whether the introduction of human p32 cDNA could complement the growth retardation in the glycerol medium. The growth retardation of the \(\Delta p30\) strain was partially restored by the introduction of p32 cDNA (Fig. 7A, \(\Delta p30 + pGAPY1\)) but not by vector alone (Fig. 7A, \(\Delta p30 + pGAP\)). The \(\Delta p30\) haploid strain was crossed with another \(\Delta p30\) haploid strain or with wild type. The growth rate of the \(\Delta p30/\Delta p30\) homozygote in glycerol medium was much slower than that of the wild type/\(\Delta p30\) heterozygote or wild type/wild type homozygote at 18 \(\degree\)C (Fig. 7B). Thus, yeast p30 deletion mutant showed cold sensitivity in the nonfermentable carbon source medium.

We determined the specific activity of succinate-cytochrome c reductase in subcellular fractions are expressed as percent of those in cytosol, mitochondrial, and microsomal fractions, respectively. Each value is a mean of two independent experiments.

**FIG. 7. Growth properties of the yeast strains.** A and B, growth properties of the indicated yeast cells on glucose (YPDagar) and glycerol (YPGagar) media are shown. Each plate was incubated at 18 \(\degree\)C for 10 days. WT, YPH499; WT/WT, the diploid strain of YPH499/YPH500; WT/\(\Delta p30\), the diploid strain of YPH500/YPH499 (\(\Delta p30::HIS3\)); \(\Delta p30::\Delta p30\), the diploid strain of YPH500 (\(\Delta p30::TRP1\)/YPH499 (\(\Delta p30::HIS3\)). C, logarithmic plot of \(A_{600}\) versus time in hours for the indicated strains grown in the glycerol medium (YPG) at 30 \(\degree\)C. Cells were first grown in the synthetic medium with glucose at 30 \(\degree\)C and then diluted into complete medium containing 3% glycerol. At the indicated times, samples were removed and their \(A_{600}\) values were determined. \(\times\), wild type strain (YPH499) carrying plasmid alone (pGAP); \(\bullet\), \(\Delta p30\) strain (YPH499 (\(\Delta p30::HIS3\))) carrying plasmid alone; \(\bigcirc\), \(\Delta p30\) strain carrying the yeast p30 plasmid (pGAPY1); \(\square\), \(\Delta p30\) strain carrying the human p32 plasmid (pGAPH1).

We have shown in the present study that p32 is localized in the mitochondrial matrix in a soluble form. p32 was not detected in membrane-associated fractions in our experiment. It seems unlikely that p32 works on plasma membrane as a receptor for C1q, H-kininogen, and hyaluronic acid. The small amount of p32 present in the nuclear fraction (Table III) may be contamination with mitochondria, because (i) the specific content of p32 in the nuclear fraction was almost the same as the specific activity of succinate-cytochrome c reductase in the nuclear fraction, (ii) nuclei were barely stained in immunocytochemistry, and (iii) p32 protein in the nuclear fraction was the mitochondrially processed mature form.

p32 protein has been assumed to be biosynthetized as a proprotein of 282 amino acids and post-transcriptionally processed to the mature protein of 209 amino acids (fragment 74–282) (18). The processing mechanisms have been unknown. Most mitochondrial proteins are synthesized with an N-terminal signal sequence, which targets these proteins to mitochon-
dria and is excised in mitochondria. A program, PSORT, predicts that the N-terminal region of premature p32 has a structure typical to mitochondrial signal peptide, i.e. amphipathic helix with basic residues. Here we show that the N-terminal region is required for the import of p32 into mitochondria and is efficiently processed (Fig. 4).

We demonstrated that yeast p30 is localized in the mitochondrial matrix, and the disruption of the gene causes the slow growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal. The cold sensitivity on the glycerol medium as was seen in the growth in glycerol medium, but the slow growth is not lethal.

The growth impairment in the glycerol medium of the p30 gene- disrupted strain was restored by the introduction of the human p32 gene to exactly the same extent as that of the yeast p30 gene. The p32 protein may play a physiologically important role in human mitochondria.

There is a report that the peripheral T lymphocytes from HIV1 carriers show dysfunction of mitochondria (41). The interaction of p32 protein with HIV Tat might affect mitochondrial functions. Although many proteins have been supposed to interact with the human p32 protein in nucleus or on plasma membrane, the interactions with p32 should be carefully re-examined from the view of its exclusive localization in mitochondria.

Acknowledgments—We thank Dr. Hideki Sumimoto (Kyushu University) for his critical reading and useful comments.

REFERENCES
1. Krainer, A. R., Mayeda, A., Kazak, D., and Birnstiel, M. (1991) Cell 66, 383–394
2. Desai, K., Loewenstein, P. M., and Green, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8875–8879
3. Yu, L., Zhang, Z., Loewenstein, P. M., Desai, K., Tang, Q., Mao, D., Symington, J. S., and Green, M. (1990) J. Virol. 64, 3067–3076
4. Fridelli, R. A., Harding, L. S., Bogerd, H. P., and Cullen, B. R. (1995) Virology 209, 347–357
5. Luu, Y., Yu, H., and Peterlin, B. M. (1994) J. Virol. 68, 3850–3856
6. Tange, T. O., Jensen, T. H., and Kjems, J. (1996) J. Biol. Chem. 271, 10066–10072
7. Yu, L., Loewenstein, P. M., Zhang, Z., and Green, M. (1995) J. Virol. 69, 3017–3023
8. Simonis, G., and Georgatos, S. D. (1994) FEBS Lett. 346, 225–228
9. Nikolakaki, E., Simonis, G., Georgatos, S. D., and Giannakourou, T. (1996) J. Biol. Chem. 271, 8365–8372
10. Ghebrehiwet, B., Lim, B. L., Peerschke, E. I. B., Willis, A. C., and Reids, K. B. M. (1992) J. Exp. Med. 176, 1809–1821
11. Lim, B.-R., Reid, K. B. M., Ghebrehiwet, B., Peerschke, E. I. B., Leigh, L. A. E., and Freissmuth, K. T. (1996) J. Biol. Chem. 271, 26739–26744
12. Dedio, J., and Esterl, W. M. (1996) J. Biol. Chem. 271, 13040–13047
13. Deb, T. B., and Datta, K. (1996) J. Biol. Chem. 272, 2062–2071
14. Deleted in proof
15. Yang, T., Zoumpas, K. M., and Craig, R. W. (1995) J. Cell Biol. 128, 1173–1184
16. Dedio, J., and Esterl, W. M. (1996) FEBS Lett. 399, 255–258
17. Kug, N., Nishida, J., Iyama, A., Nakaseputu, Y., Furuichi, M., Fujiwara, T., Sataguchi, M., and Takeshige, K. (1995) J. Biol. Chem. 270, 14659–14665
18. Horone, B., Madeen, P., Rasmussen, H. V., Vandekerkenkove, J., Cels, J. E., and Leffers, H. (1993) Gene (Amst.) 134, 283–287
19. Tanaka, K., Matsumoto, K., and Tobe, A. (1988) EMBO J. 7, 495–502
20. Seebacher, T., and Bade, E. G. (1996) Electrophoresis 17, 1573–1574
21. Becker, A., Reith, A., Napiwotzki, J., and Kadenbach, B. (1996) Anal. Biochem. 237, 204–207
22. Larsson, S. H., Charlton, J. P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., Heyningen, V., and Hastie, N. D. (1995) Cell 81, 391–401
23. Kamura, T., Handa, H., Hamasaki, N., and Kitaizumi, S. (1997) J. Biol. Chem. 272, 11361–11368
24. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1988) J. Bacteriol. 153, 163–168
25. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Yamazaki, S., Harashima, S., Sakaguchi, M., and Mihara, K. (1997) J. Biochem. (Tokyo) 121, 8–14
27. Pearson, W. R. (1996) Methods Enzymol. 266, 227–228
28. Rothenstein, R. (1991) Methods Enzymol. 194, 281–301
29. Ward, E. R., and Jen, G. C. (1990) Methods Enzymol. 188, 736–741
30. Baudin, A., Kalogeropoulos, O., Denoue, A., Lecroart, F., and Cullin, C. (1993) Biochimie 75, 329–3330
31. Kondo, K., and Inouye, M. (1991) J. Biol. Chem. 266, 17537–17544
32. McKee, E. E., and Poyton, R. O. (1984) J. Cell Biol. 99, 9520–9531
33. Daum, G., Bohni, P. C., and Schatz, G. (1982) J. Biol. Chem. 257, 13038–13043
34. Bergmeyer, H. U., Bernt, E., and Hess, B. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 736–741, Academic Press, NY
35. Blumenthal, C., and Greenawalt, J. W. (1968) J. Biol. Chem. 243, 158–157
36. Masters, B. S. K., Kamion, H., Gibson, Q. H., and Williams, C. H., Jr. (1965) Proc. Natl. Acad. Sci. U. S. A. 53, 265–275
37. Cell 89, 903–913
38. Singh, B., Patel, H. V., Ridely, B. G., Freeman, K. B., and Gupta, R. S. (1990) Biochem. Biophys. Res. Commun. 169, 391–396