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A Polypeptide Derived from Mitochondrial Dihydrolipoamide Succinyltransferase Is Located on the Plasma Membrane in Skeletal Muscle

Sadayuki Matuda,*[,1] Junko Kodama,* Norihisa Goshi,* Chiyoshi Takase,†
Kyoko Nakano,† Shiro Nakagawa,‡ and Shigeo Ohta§[,1]

**Department of Biology and Health Science, Kanoya National Institute of Fitness and Sports, Kanoya, Kagoshima 891-23, Japan; †Department of Biochemistry, Kagoshima Women's Junior College, Kagoshima 890, Japan; ‡Department of Anatomy, School of Medicine, Kagoshima University, Kagoshima 890, Japan; and §Department of Biochemistry and Cell Biology, Institute of Gerontology, Nippon Medical School, Kawasaki, Kanagawa 211, Japan*

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Dihydrolipoamide succinyltransferase (DLST) is the core-enzyme of 2-oxoglutarate dehydrogenase complex which is located in mitochondria. In this study, several tissues from rat and human were immunostained with an affinity-purified anti-DLST antibody. Of the tissues examined, the plasma membrane of skeletal muscle was immunostained with the antibody besides mitochondria. Furthermore, subcellular fractionation analysis coupled with Western blotting demonstrated that the antigen of the anti-DLST antibody is distributed on the plasma membrane fraction in addition to the mitochondria fraction in skeletal muscle and that it is free from the complex. The molecular weight of the polypepetide bound to the plasma membrane was about 20 kilodaltons (kDa). The polypeptide was purified by immunoprecipitation and its N-terminal amino-acid sequence was determined. The amino-acid sequence exactly corresponded to a part of DLST. Northern blots revealed the presence of mRNA corresponding to the 20 kDa protein. We are the first to report that a mitochondrial protein is also present on the plasma membrane in skeletal muscle as well as in mitochondria.

The 2-oxoglutarate dehydrogenase complex is located in mitochondria and converts 2-oxoglutarate to succinyl-CoA. The complex is composed of three enzymes: 2-oxoglutarate decarboxylase, dihydrolipoamide succinyltransferase (DLST) and dihydrolipoamide dehydrogenase (1-4). The core of the complex is constructed by the self-assembly of multiple copies of DLST, to which multiple copies of the 2-oxoglutarate decarboxylase and dihydrolipoamide dehydrogenase noncovalently bind. The cDNAs for DLST have been isolated from human and rat and sequenced (5, 6). The human DLST genomic gene has been also isolated and its sequence has been determined (7, 8). We have recently found that a genotype of the DLST gene based on polymorphic variations is closely associated with Alzheimer's disease (9). Therefore, an unusual DLST molecule is expected to be a candidate that contributes to the pathogenesis of Alzheimer's disease.

So far, DLST has been found only in mitochondria among intracellular organella. In this study, several rat and human tissues were immunostained with anti-DLST antibody. We found that of several tissues examined, the plasma membrane of skeletal muscle was immunostained with the anti-DLST antibody in addition to the immunostaining of mitochondria. Furthermore, subcellular fractionation analysis demonstrated the presence of the antigen on the plasma membrane of the skeletal muscle. Furthermore, the polypeptide was confirmed to be derived from DLST by amino-acid sequencing. These results indicate that the DLST gene encodes the membrane bound 20 kDa enzyme with an unknown function, as well as DLST that functions a part of the complex.

MATERIALS AND METHODS

Preparation of antibodies. Anti-pyruvate dehydrogenase complex and anti-2-oxoglutarate dehydrogenase complex antibodies were raised in rabbits against the complexes purified from the rat heart as described (5, 10). The antibody specific for DLST was prepared as follows: The 2-oxoglutarate dehydrogenase complex purified from rat heart was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (immobilon P, Millipore). After reacting the band of DLST on an immobilon P

[1] To whom correspondence may be addressed. (S.M.), Fax: +81-994-46-4947. E-mail: matsuda@nifs-k.ac.jp. (S.O.). Fax: +81-44-733-1877. E-mail: ohta@nms.ac.jp.

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membrane with the anti 2-oxoglutarate dehydrogenase complex antibody, the antibody that reacted with DLST was extracted with 0.1 M glycine-acetate buffer (pH 3.0) and used as the specific antibody against rat DLST. Anti ATP synthase β-subunit antibody was prepared as described (11).

Immunocytochemical staining. The rat skeletal muscle was examined. Rats were deeply anesthetized and perfused through the ascending aorta with 1% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylic acid buffer, pH 7.4. The skeletal muscle was then removed and post-fixed in the same fixative. The skeletal muscle embedded in paraffin was cut on a microtome at 7 μm thickness. The sections were incubated for 4 days with the primary rabbit antibodies (anti-DLST antibody at a concentration of 1 μg/ml in 0.1 M phosphate buffer, pH 7.4; anti-pyruvate dehydrogenase complex and anti-ATP synthase β-subunit antisera at a 1:2,000 dilution in the same buffer) at 4 °C. After several rinses with the same buffer, the sections were incubated for 2 days with goat anti-rabbit IgG (EY Laboratories, Inc, San Metro, USA) at a 1:2,000 dilution as a secondary antibody at 4 °C. After several rinses with 0.1 M phosphate buffer, pH 7.4, the sections were incubated for 1 day with peroxidase conjugated anti-peroxidase (rabbit PAP, DAKO A/S, Denmark). After several rinses with 0.1 M phosphate buffer, pH 7.4, the peroxidase reaction was developed using 3,3′-diaminobenzidine as a substrate. Human skeletal muscle obtained by autopsy within 5 h after death, were embedded in paraffin. These were cut into sections 4 μm thick and immunostained as described for rat skeletal muscle.

Assays of enzyme activities. The overall activity of the 2-oxoglutarate dehydrogenase complex was determined by monitoring NADH formation at 340 nm at 25 °C with a recording spectrophotometer taking the millimolar extinction coefficient of NADH as 6.2 as reported (5). Leucine aminopeptidase activity was measured as reported (12) using L-leucyl-3-carboxy-4-hydroxyanilide as a substrate instead of L-leucyl-β-naphthylamide. Succinate-cytochrome *c* reductase activity was measured according to the methods of Stotz (13).

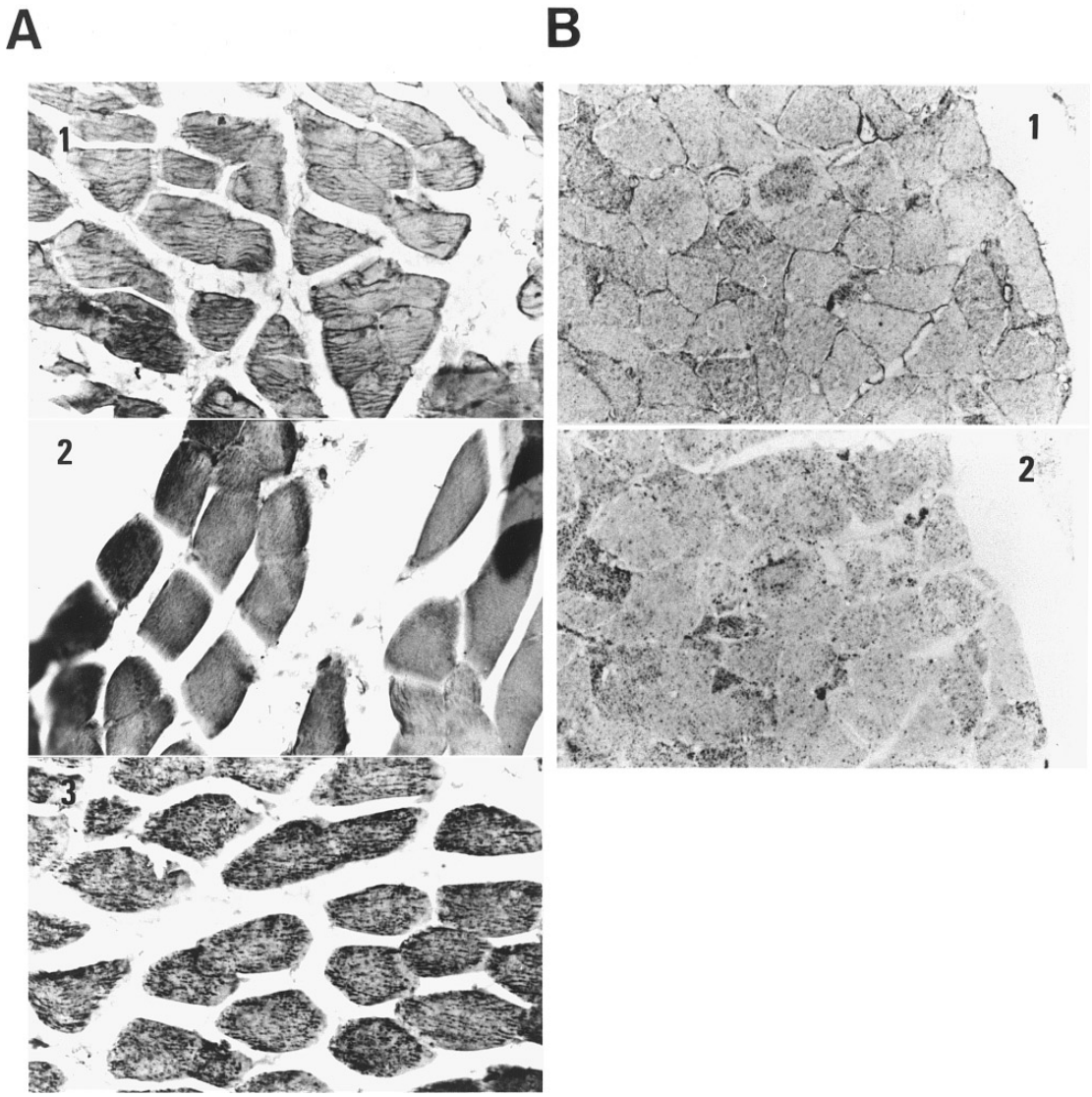

FIG. 1. Immunocytochemical staining of rat and human skeletal muscle with affinity purified anti DLST antibody. A, rat skeletal muscle; pictures 1, 2, and 3 indicate sections stained with anti-DLST antibody, anti-pyruvate dehydrogenase complex antibody, and anti-ATP synthase β-subunit antibody, respectively. B, human skeletal muscle; pictures 1 and 2 indicate sections stained with anti-DLST antibody and anti-pyruvate dehydrogenase complex antibody, respectively. Magnification, ×300.

Subcellular fractionation. Rat skeletal muscle was pulverized with a razor and homogenized in 3 volumes of 0.25 M sucrose containing 5 mM Tris-HCl and 0.1 mM EDTA, pH 7.4 with a Teflon homogenizer. The homogenate was centrifuged at 900 × *g* for 5 min and the supernatant was centrifuged at 10,000 × *g* for 15 min to obtain the mitochondrial fraction. The post-mitochondrial supernatant was further centrifuged at 105,000 × *g* for 2 h, and the precipitate was recovered as the microsomal fraction. The supernatant was used as the soluble cytosolic fraction.

Western blot. Mitochondrial and microsomal fractions were resolved by SDS-PAGE. The gel was then electroblotted onto an immobilon P membrane, then reacted with anti-2-oxoglutarate dehydrogenase complex antibody or anti-DLST antibody. After washing, the membrane was reacted with the peroxidase conjugated secondary antibody and detected with 3,3′-diaminobenzidine.

Purification of the polypeptide and amino-acid sequencing. The small polypeptide bound to anti-DLST antibody was purified by immunoprecipitation as follows. Rat (8 week-year-old) skeletal muscle (5 g) of thigh was homogenized in 3 volumes of A-buffer (0.1 mM EDTA, 5 mM 2-mercaptoethanol and 5 mM Tris-HCl, pH 7.5) containing 0.25 M sucrose and the plasma membrane was obtained in the supernatant by centrifugation at 10,000 × *g* for 10 min and centrifuged at 105,000 × *g* for 1 h. The polypeptide was dissolved from the plasma membrane fraction in 5 ml of A-buffer containing 1.5% Triton X-100 and 500 mM NaCl and insoluble materials were removed by centrifugation at 105,000 × *g* for 1 h. Anti rabbit serum against the rat 2-oxoglutarate dehydrogenase complex was incubated with the supernatant for 3 h on ice. The resulting immunocomplex was precipitated by centrifugation at 10,000 × *g* for 5 min and washed 5 times with A-buffer containing 1.5% Triton X-100 and 500 mM NaCl, and then resolved by SDS-PAGE. The proteins were electroblotted onto a PVDF membrane and stained with Comassie blue. A band corresponding to 20 kDa was cut-off and applied to a Hewlett Packard protein sequencer G1005A to determine amino acid sequence.

Northern blot. Total RNA from rat skeletal muscle was denatured with formaldehyde, separated by 1% agarose gel electrophoresis, and blotted onto a nitrocellulose memberane as described (14). The blot was analyzed with nick-translated, ^{32}P-labelled rat DLST cDNA (5) under reported hybridization condition (14).

RESULTS AND DISCUSSION

Immunocytochemical staining of skeletal muscle with anti DLST antibody. When several tissues were stained with an affinity purified anti-DLST antibody, the profile in skeletal muscle was unusual. Figure 1 shows the immunocytochemical staining of rat skeletal muscle with the anti-DLST antibody. Immunocytochemical staining with anti-pyruvate dehydrogenase complex and ATP synthase β-subunit antibodies is also shown for comparison (FIG. 1). The pyruvate dehydrogenase complex and the ATP synthase β-subunit are also localized in mitochondria and play significant roles in energy productive metabolism. As shown in FIG. 1, the anti-DLST antibody immunostained the plasma membrane of skeletal muscle as well as the mitochondria. In contrast to the staining with the anti-DLST antibody, the anti pyruvate dehydrogenase complex and ATP synthase β-subunit antibodies did not stain the plasma membrane of skeletal muscle.

FIG. 1 also shows the immunocytochemical staining of human skeletal muscle with the anti-DLST and -pyruvate dehydrogenase complex antibodies. The anti-DLST antibody immunostained the plasma membrane of human skeletal muscle, whereas the anti-pyruvate dehydrogenase complex antibody did not. In this study, we examined the heart, kidney, smooth muscle of intestine, spinal cord and thyroid gland with the anti-DLST antibody. However, the plasma membranes of these tissues were not immunostained. Although the immunostaining of the plasma membrane with the anti DLST antibody was far less than that of the mitochondria in rat and human skeletal, this study suggested that DLST or a DLST related polypeptide is present not only in the mitochondria, but also the plasma membrane in skeletal muscle.

Biochemical characterization of mitochondrial and microsomal fractions of rat skeletal muscle. We characterized the plasma membrane bound antigen in subcellular fractions of rat skeletal muscle, as described in MATERIALS AND METHODS. Succinate-cytochrome *c* reductase and leucine aminopeptidase were used as marker enzymes for the mitochondria and the plasma membrane (11), respectively. The succinate-cytochrome *c* reductase and 2-oxoglutarate dehydrogenase complex activities were mainly found in the mitochondrial fraction at eight-fold higher levels than those in the microsomal fraction, while the leucine aminopeptidase activity was mainly located in the microsomal fraction at a nine-fold higher level than that in the mitochondrial fraction (Table I). These results confirmed that this microsomal fraction contained the plasma membrane of skeletal muscle as expected.

Detection of plasma membrane bound antigen. To detect the plasma membrane bound antigen, the microsomal fraction of rat skeletal muscle was resolved by SDS-PAGE, blotted onto the membrane and then stained with the anti-2-oxoglutarate dehydrogenase complex or the anti-DLST antibodies (FIG. 2). When the anti-2-oxoglutarate dehydrogenase complex antibody was used, two bands of 2-oxoglutarate decarboxylase (M.W; 110,000) and DLST (M.W; 48,000) were found in mitochondrial fraction (FIG. 2A, lanes 1 and 4). In addition, an additional band appeared in the microsomal fraction and its molecular weight was about 20 kilodaltons (kDa) (FIG. 2A, lane 2). No band was found in the soluble cytosolic fraction after ultracentrifugation at 105,000 σ *g* (FIG.2A, lane 3). Using the anti-DLST antibody, only a single band corresponding to DLST (48 kDa) was found in the mitochondrial fraction (FIG. 2B, lane 2), while the polypeptide with a molecular weight 20 kDa was also found in the microsomal fraction (FIG. 2B, lane 1). No band was found in the soluble cytosolic fraction (FIG. 2B, lane 3). The applied amounts of the mitochondrial and microsomal fractions on SDS-poly-

TABLE I

Distribution of Succinate-Cytochrome *c* Reductase, Leucine Aminopeptidase, and 2-Oxoglutarate Dehydrogenase Complex Activities in Homogenate, Mitochondrial, and Microsomal Fractions from Rat Skeletal Muscle

	Succinate-cytochrome *c* reductase activity (μmole/min/g tissue)	Leucine aminopeptidase activity (μmole/min/g tissue)	2-oxoglutarate dehydrogenase complex activity (μmole/min/g tissue)
Homogenate	103.5 (100%)	33.1 (100%)	137.5 (100%)
Mitochondrial fraction	73.4 (71%)	2.6 (8%)	99.4 (72%)
Microsomal fraction	9.2 (9%)	23.1 (70%)	12.2 (9%)

Note. Values in parentheses are percentages based on the amounts in total homogenate.

acrylamide gel were 1.3:1 in terms of 2-oxoglutarate dehydrogenase complex activity.

The microsomal fraction being treated with Triton X-100 and centrifuged at 105,000 × *g* for 2 h, the 20 kDa polypeptide would be released from the plasma membrane of skeletal muscle and recovered in the supernatant if it binds to the plasma membrane. On contrary, the 2-oxoglutarate dehydrogenase complex is expected to be recovered in the precipitate because it is known to be precipitated by centrifugation at 105,000 × *g* for 2 h (5). Therefore, the microsomal fraction of rat skeletal muscle was solubilized with Triton X-100 and centrifuged at 105,000 × *g* for 2 h. The supernatant and precipitate were analyzed by Western blotting using the anti-2-oxoglutarate dehydrogenase complex and anti-DLST antibodies (FIG. 3). When the 2-oxoglutarate dehydrogenase complex antibody was used in the Western blot, the 20 kDa protein was found in the supernatant solubilized with Triton X-100 (FIG. 3A, lane 2). In contrast, 2-oxoglutarate decarboxylase and DLST were found in the precipitate (FIG. 3A, lane 1). These results suggested that the 20 kDa polypeptide is bound to the plasma membrane and free from the complex. These results were also supported by the results of the Western blot with the DLST antibody (FIG. 3B).

Amino acid sequencing of the polypeptide. To confirm whether the polypeptide is derived from the DLST gene product, we determined the amino acid sequence of the polypeptide purified by immunoprecipitation as

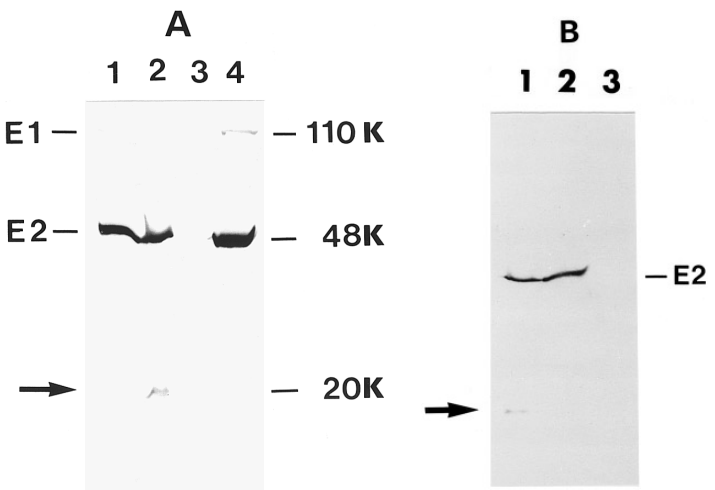

FIG. 2. Western blots of the mitochondrial and microsomal fractions of rat skeletal muscle with anti-2-oxoglutarate dehydrogenase complex and anti-DLST antibodies. A, Western blot with anti-2-oxoglutarate dehydrogenase complex antiserum (1:3,000 dilution in 20 mM Tris-HCl, 500 mM M NaCl, pH 7.4). Lanes 1 and 4, mitochondrial fraction (lane 1, 23 μg of protein, containing 3.8 μmoles/min of 2-oxoglutarate dehydrogenase complex activity; lane 4, 46 μg of protein); lane 2, microsomal fraction (225 μg of protein, containing 3 μmoles/min of 2-oxoglutarate dehydrogenase complex activity); lane 3, soluble cytosolic fraction (140 μg of protein, no 2-oxoglutarate dehydrogenase complex activity was detected). B, Western blot with the anti-DLST antibody (at a final concentration of 0.5 μg/ml in 20 mM Tris-HCl, 500 mM NaCl, pH 7.4). Lane 1, microsomal fraction (225 μg of protein); lane 2, mitochondrial fraction (23 μg of protein); lane 3, soluble cytosolic fraction (14 μg of protein). E1 and E2 indicate 2-oxoglutarate decarboxylase (M.W; 110 kDa) and DLST (M.W; 48 kDa), respectively. Arrows indicate the 20 kDa protein in the microsomal fraction of rat skeletal muscle.

described in MATERIALS AND METHODS. The N-terminal amino acid sequence was determined to be X-A-A-P-E-A-P-A-A-P-P-P (X: undetermined amino acid residue), which is identical to 154-164 amino acid residues of rat DLST (6). Thus, the polypeptide bound to the plasma membrane was confirmed to be derived from the DLST gene product.

Northern blot. The 20 kDa polypeptide may be produced by alternative splicing of the DLST gene. If so, there would be an mRNA species for this protein in addition to the 2.8 kb mRNA for the mitochondrial DLST in Northern blot. We examined total RNA from rat skeletal muscle by Northern blot using rat DLST cDNA as a probe (FIG. 4). One major 2.8 kb band corresponding to the mitochondrial DLST (5) and a minor band of 1.2 kb were visualized.

The ratio of DLST and 20 kDa polypeptide in rat skeletal muscle was about 80:1 based upon the 2-oxoglutarate dehydrogenase complex activity and the intensities of bands of both proteins analyzed by Western blotting. Therefore, the relative intensity of the 1.2 kb band in the Northern blot is enough to explain an amount of the 20 kDa polypeptide. In addition, the 1.2 kb mRNA is long enough to encode the 20 kDa polypeptide.

Since the anti-DLST antibody used in this study was highly purified as described in MATERIALS AND METHODS, the possibility of contaminating antibodies against unrelated antigens would be excluded. There was no significant homologous sequence with any other plasma membrane proteins when DLST was compared with other known plasma membrane proteins by a homology search. Thus, it is unlikely that a muscle-specific protein with the common antigenisity exists on the plasma membrane. In fact, the N-terminal amino acid sequence of the polypeptide exactly corresponded to a part of DLST. As an origin of the 20 kDa polypeptide, degradation of DLST during the fractionation procedure cannot explain the immunostaining of the plasma membrane of skeletal muscle with the anti-DLST antibody. Therefore, the muscle 20 kDa polypeptide on the plasma membrane should be a product of the DLST gene.

The 20 kDa polypeptide may be generated by alternative splicing and mistargetted to the plasma membrane of skeletal muscle. It is, however, necessary to confirm this speculation by isolating cDNA for the 20 kDa polypeptide in future. The function of the 20 kDa polypeptide on the plasma membrane of skeletal muscle is unknown and remains to be resolved. We have recently found that a genotype of the DLST gene is highly associated with Alzheimer's disease (9). The DLST polypeptide bound to the plasma membrane may be accumulated in the brains of patients with Alzheimer's disease. Further study is required to reveal the relation of the DLST gene product with the disease in future.

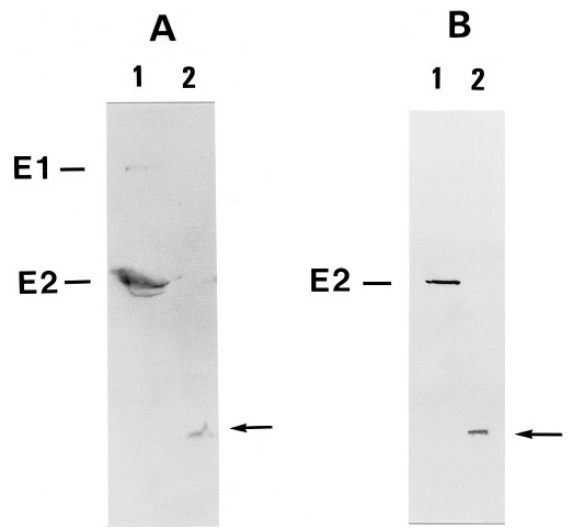

FIG. 3. Release of the 20 kDa protein from the plasma membrane of rat skeletal muscle using Triton X-100. The microsomal fraction was prepared as described in MATERIALS AND METHODS. The microsomal fraction (3.95 ml) was solubilized with a final concentration of 1% Triton X-100 and centrifuged at 105,000 × *g* for 2 h. The precipitate (resolved with 3.5 ml of 0.25 M sucrose, 5 mM Tris-HCl, and 0.1 mM EDTA, pH 7.4) and the supernatant (3.7 ml) were Western blotted using antiserum against anti-2-oxoglutarate dehydrogenase complex (1:3,000 dilution in 20 mM Tris-HCl, 500 mM NaCl, pH 7.4) (for A) and anti-DLST antibody (final concentration of 0.5 μg/ml in 20 mM Tris, 500 mM NaCl, pH 7.4) (for B). Lanes 1 and 2 indicate samples from the precipitate (110 μg of protein) and the supernatant (200 μg of protein), respectively.

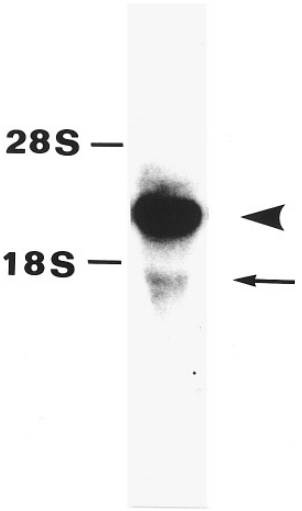

FIG. 4. Northern blot of rat skeletal muscle RNA. Total RNA from rat skeletal muscle (50 μg) was probed with nick-translated rat DLST cDNA (5). The arrowhead indicates the 2.8 kb mRNA corresponding to DLST and the arrow an mRNA species of 1.2 kb.

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