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Molecular Cloning of cDNA Encoding a 55-kDa Multifunctional Thyroid Hormone Binding Protein of Skeletal Muscle Sarcoplasmic Reticulum*

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A cDNA clone encoding 55-kDa multifunctional, thyroid hormone binding protein of rabbit skeletal muscle sarcoplasmic reticulum was isolated and sequenced. The cDNA encoded a protein of 509 amino acids, and a comparison of the deduced amino acid sequence with the NH_2-terminal amino acid sequence of the purified protein indicates that an 18-residue NH_2-terminal signal sequence was removed during synthesis. The deduced amino acid sequence of the rabbit muscle clone suggested that this protein is related to human liver thyroid hormone binding protein, rat liver protein disulfide isomerase, human hepatoma β-subunit of prolyl 4-hydroxylase and hen oviduct glycosylation site binding protein. The protein contains two repeated sequences Trp-Cys-Gly-His-Cys-Lys proposed to be in the active sites of protein disulfide isomerase. Northern blot analysis showed that the mRNA encoding rabbit skeletal muscle form of the protein is present in liver, kidney, brain, fast- and slow-twitch skeletal muscle, and in the myocardium. In all tissues the cDNA reacts with mRNA of 2.7 kilobases in length.

The 55-kDa multifunctional thyroid hormone binding protein was identified in isolated sarcoplasmic reticulum vesicles using a monoclonal antibody specific to the 55-kDa thyroid hormone binding protein from rat liver endoplasmic reticulum. The mature protein of M_r 56,681 contains 95 acidic and 61 basic amino acids. The COOH-terminal amino acid sequence of the protein is highly enriched in acidic residues with 17 of the last 29 amino acids being negatively charged. Analysis of hydropathy of the mature protein suggests that there are no potential transmembrane segments. The COOH-terminal sequence of the protein, Arg-Asp-Glu-Leu (RDEL), is similar to but different from that proposed to be an endoplasmic reticulum retention signal; Lys-Asp-Glu-Leu (KDEL) (Munro, S., and Pelham, H. R. B. (1987) *Cell* 48, 899–907). This variant of the retention signal may function in a similar manner to the KDEL sequence, to localize the protein to the sarcoplasmic or endoplasmic reticulum. The positively charged amino acids Lys and Arg may thus interchange in this retention signal.

In muscle tissue the sarcoplasmic reticulum is a highly specialized form of the endoplasmic reticulum. It plays a key role in the regulation of the contraction-relaxation cycle by controlling the levels of free cytoplasmic calcium (1). The membrane is composed of several integral and peripheral proteins (2). The major integral protein of the sarcoplasmic reticulum is the 110-kDa Ca^{2+}-ATPase, which removes calcium from the cytosol causing a decrease in free calcium concentration and initiating relaxation (1, 2). This protein constitutes about 60–70% of the sarcoplasmic reticulum proteins. Calcium removed from the cytosol by the Ca^{2+}-ATPase is stored within the sarcoplasmic reticulum and is likely bound to calsequestrin, a major peripheral calcium binding protein present in the lumen of the sarcoplasmic reticulum (3). In addition to calsequestrin (4, 5) a number of sarcoplasmic reticulum peripheral membrane proteins have recently been cloned including 160-kDa sarcolumenin (6) and 53-kDa glycoproteins (7), the 165-kDa histidine-rich protein, calcium and low density lipoprotein binding protein (HCP) (8), and calreticulin (9).

Calreticulin is a calcium binding, peripheral membrane protein identified first in the skeletal muscle sarcoplasmic reticulum as the high affinity calcium binding protein (10). In our recent studies we have discovered that calreticulin is a protein common to both sarcoplasmic reticulum of muscle cells and endoplasmic reticulum of non-muscle cells (11). Analysis of the amino acid sequence deduced from the cDNA encoding calreticulin revealed that the protein terminates with a long stretch of acidic residues (37 out of last 56) followed by the Lys-Asp-Glu-Leu (KDEL) retention signal (9, 11–13). This is similar to a number of peripheral membrane proteins present in the endoplasmic reticulum including the 55-kDa multifunctional thyroid hormone binding protein (T_3BP),[1] some heat shock proteins and some glucose-regulated proteins (14). These proteins have not been identified in the sarcoplasmic reticulum in muscle cells. Recently, we have discovered that T_3 binds to the solubilized cardiac and skeletal muscle sarcoplasmic reticulum membranes (14), in-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05602.

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[1] The abbreviations used are: T_3BP, thyroid hormone binding protein; PDI, protein disulfide isomerase; T_3, 3,5,3′-triiodo-L-thyronine; SDS, sodium dodecyl sulfate; bp, base pairs; kb, kilobases.

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dicating that the 55-kDa multifunctional T_3BP may be present in the sarcoplasmic reticulum membrane.

It has recently been shown that a single 55-kDa polypeptide may be a multifunctional protein with the activities of protein disulfide isomerase (PDI) (15), glycosylation site binding protein of oligosaccharyl transferase (16), the β-subunit of prolyl 4-hydroxylase (17), and T_3BP (18, 19). All of these proteins have shown homology in their amino acid sequences and predicted protein structures (16, 20–24). They are localized to the lumen of the endoplasmic reticulum (16, 19–21, 25, 26) and some of them form a common subunit of a larger protein complex such as prolyl 4-hydroxylase and oligosaccharyl transferase. The sequence similarities are very extensive as the coding region of the 55-kDa human T_3BP cDNA (21, 23) has an 85% sequence similarity to rat PDI (22) and 98% sequence similarity to the β-subunit of human prolyl 4-hydroxylase (24). All four proteins terminate with the KDEL COOH-terminal amino acid sequence. This sequence has previously been proposed to be an endoplasmic reticulum retention signal (27, 28), supporting their lumenal localization in the endoplasmic reticulum membrane. A number of experiments support the concept that these proteins are identical and are the product of the same gene. Antibody to human T_3BP cross-reacts with hen glycosylation site binding protein (16). A photoaffinity probe that recognizes glycosylation site binding protein labels purified PDI (16) and the β subunit of prolyl 4-hydroxylase and PDI exhibit the same peptide maps and immunoreactivity (29). The β subunit of prolyl 4-hydroxylase (29) and purified bovine T_3BP (30) have also been shown to possess PDI activity.

The functions of these four related proteins are different. PDI is involved in rearrangement of disulfide bonds of newly synthesized proteins (15). Prolyl 4-hydroxylase catalyzes the formation of 4-hydroxyproline in newly synthesized collagen (31) and glycosylation site binding protein is a component of oligosaccharyl transferase which catalyzes the transfer of oligosaccharide chains from dolicholpyrophosphoryloligosaccharide to asparagine residues of proteins (32). If indeed all four activities belong to one and the same protein then it is possible that T_3 binding to this endoplasmic reticulum protein may be the mechanism through which the hormone effects the translation and/or modification of newly synthesized proteins.

In this report we have identified and isolated a full length cDNA clone encoding the rabbit slow-twitch skeletal muscle form of the sarcoplasmic reticulum T_3BP. We report the deduced amino acid sequence of the clone and show that it is homologous to PDI, the β subunit of prolyl 4-hydroxylase, glycosylation site binding protein and T_3BP. The COOH-terminal sequence of the protein terminates with the residues Arg-Asp-Glu-Leu-COOH (RDEL), a variant of the Lys-Asp-Glu-Leu-COOH (KDEL) retention signal. Using a monoclonal antibody against the rat liver endoplasmic reticulum T_3BP we have identified this protein in isolated skeletal muscle sarcoplasmic reticulum vesicles. For the sake of clarity, throughout this paper, we refer to this protein as the 55-kDa multifunctional T_3BP.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim and Bethesda Research Laboratories. Erase-a-base exonuclease kit was purchased from Promega. The plasmids pTZ18R and 19R were from Pharmacia LKB Biotechnology inc. [α-^{32}P]ATP and [α-^{35}S]ATP were obtained from Du Pont-New England Nuclear. Nitrocellulose membrane filters were from Schleicher & Schuell and nylon membranes were from Amersham Corp. Polyvinylidene difluoride membranes were from Millipore. Sequencing gels were prepared using reagents from Bio-Rad or Boehringer Mannheim. SDS-polyacrylamide gel electrophoresis reagents and molecular weight markers were purchased from Bio-Rad. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Boehringer Mannheim. All chemicals were of the highest grade available.

Screening and Sequencing of cDNA Clones—The λgt11 cDNA expression libraries were constructed from poly(A)$^+$ RNA of rabbit slow-twitch (soleus) muscle as described previously (9). The Okayama-Berg cDNA library from rabbit neonatal fast-twitch muscle was as described earlier (9). The initial 1.7-kb clone (clone 1) encoding the COOH-terminal 316 amino acid residues of the T_3BP was isolated by screening a rabbit slow-twitch muscle λgt11 expression library with affinity purified polyclonal antibodies against rabbit fast-twitch skeletal muscle calreticulin (9). This antibody reacts against the acidic COOH-terminal amino acid sequence of calreticulin (9) which is similar to the COOH-terminal amino acid sequence of T_3BP (Fig. 1). The COOH-terminal amino acid sequences of T_3BP and calreticulin also exhibit similarity with an overall charge of −14 and −15, respectively. Although useful for the isolation of clone 1, anti-calreticulin antibody did not cross-react with a partially purified rabbit skeletal muscle or rabbit liver T_3BP as analyzed by Western blotting (data not shown). A second larger 2.4-kb cDNA clone (clone 2) was isolated from the rabbit neonatal fast-twitch skeletal muscle Okayama-Berg cDNA library by screening with a 650-bp 5′ end fragment of the clone 1. The 5′ end fragment of clone 2 was used to isolate a full length 2.7-kb cDNA clone (clone 3) from a different rabbit slow-twitch muscle λgt11 cDNA library. This cDNA was subcloned into the plasmids pTZ18R and 19R and characterized by restriction endonuclease mapping. Single-stranded DNA templates were made of exonuclease treated cDNA fragments, subcloned into pTZ18R and 19R in both directions, and sequenced by the dideoxy method of Sanger *et al.* (33). The average deletion with the exonuclease was approximately 250 bp in each direction.

Northern Blot Analysis—Total and poly(A)$^+$ RNAs were isolated from various rabbit tissues (34) and 10-μg samples of each RNA and were separated by electrophoresis in denaturing formaldehyde-0.8% agarose gel stained with ethidium bromide and blotted onto nitrocellulose or nylon membranes (35). Hybridization was with ^{32}P-labeled, nick-translated cDNA fragments as described earlier (9).

Identification of a 55-kDa Multifunctional T_3BP in Sarcoplasmic Reticulum Membrane with Specific Antibody—Sarcoplasmic reticulum vesicles were isolated from rat skeletal muscle as described earlier (36) and were a generous gift of Dr. A. O. Jorgensen (University of Toronto). Rat liver endoplasmic reticulum vesicles were isolated according to Croze and Morre (37) as modified by Vance and Vance (38) and were a generous gift of Dr. J. Vance (University of Alberta). SDS-polyacrylamide gel electrophoresis was on 10% polyacrylamide gels as described by Laemmli (39). Molecular weight standards were: myosin, M_r 200,000; β-galactosidase, M_r 116,300; phosphorylase *b*, M_r 97,400; bovine serum albumin, M_r 66,000; and ovalbumin, M_r 42,000. Antibodies were screened by immunoblotting (40) after electrophoretic transfer onto nitrocellulose membrane according to the method of Towbin *et al.* (41). Monoclonal antibody produced against rat liver endoplasmic reticulum T_3BP (generous gift of Dr. S. Cheng, NIH) was used at the concentration of 40 μg of protein/ml (26). Horseradish peroxidase-conjugated goat anti-mouse IgG was used as a second antibody at the dilution of 1:2,000. Protein bands reactive with antibodies were visualized by developing a peroxidase reaction.

Miscellaneous—All other recombinant techniques were conducted according to standard protocols (35). The amino acid sequence of T_3BP was analyzed for hydropathy and secondary structure (42–44) using the Protein Works program and theoretical isoelectric point was calculated using the IBI Pustell sequence analysis programs. NH_2-terminal sequence analysis of the 55-kDa multifunctional protein was carried out using DEAE-Sepharose partially purified rabbit skeletal muscle protein (45) separated on SDS-polyacrylamide gel electrophoresis (46) and transferred to polyvinylidene difluoride membranes (41). Automated protein sequence analysis was carried out as described earlier (47). Protein was determined by the method of Lowry *et al.* (48).

RESULTS

Isolation and Analysis of cDNA Encoding the 55-kDa Multifunctional T_3BP—Using the polyclonal antibody against calreticulin (11), we isolated from a rabbit slow-twitch muscle λgt11 expression library a partial length cDNA clone (clone

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                490          500          509
T3BP  DEDGLEDLEE AEEPDLEEDD DQKAVRDEL-COOH
CRT   DDKEDEDEDE EDKDEEEEEA AAGQAKDEL-COOH
                399          409          418
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FIG. 1. **COOH-terminal sequences of T_3BP and calreticulin (*CRT*).** COOH-terminal 29 amino acids are presented based on the amino acid sequences deduced from cDNA clones encoding T_3BP (this work) and calreticulin (9). Polyclonal antibody used in this study recognizes this COOH-terminal amino acid sequence of calreticulin (9, 11, 12). Residue numbers are provided.

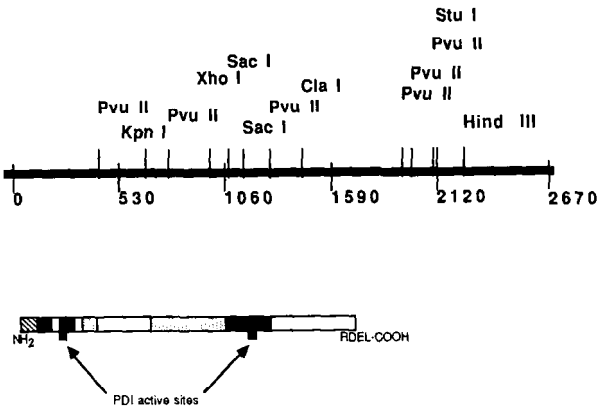

FIG. 2. **Restriction endonuclease map of a cDNA clone and predicted protein structure of rabbit slow-twitch skeletal muscle T_3BP.** A full length cDNA clone encoding T_3BP was obtained as described under "Experimental Procedures." The predicted protein structure is based on the hydropathy analysis presented in Fig. 4. The *hatched bar* represents the signal peptide; the *darkened bars* indicate potential α-helical regions, the *dotted bars* indicate potential β sheet like regions. The location of PDI active sites is indicated.

1), encoded for 1.7 kb of multifunctional T_3BP. Although the antibody used in this study was made against calreticulin (9, 11) there is considerable homology in the acidic COOH-terminal region of calreticulin and T_3BP (Fig. 1). Thus, we were able to isolate clone 1 encoding multifunctional T_3BP, which is clearly different from calreticulin. Clone 1 was used to isolate another longer cDNA clone, clone 2, from a rabbit fast-twitch skeletal muscle cDNA library, and this was used to isolate the final full length cDNA clone (clone 3) from another rabbit slow-twitch skeletal muscle library. Restriction endonuclease mapping and partial sequencing of clones 1 and 2 and the full length clone showed that these clones were identical to the full length clone, with the exception of being truncated at their 5′ ends (data not shown).

Fig. 2 shows the restriction map for the cDNA encoding the rabbit slow-twitch muscle form of multifunctional T_3BP. The full length clone contained 2670 bp of which 1604 encoded a protein of 509 amino acids. The 5′-untranslated region is 77 bp long and the 3′-untranslated sequence is 1066 bp long. No polyadenylation signals were detected within these sequences which is similar to that described for a cDNA encoding the β-subunit of human prolyl 4-hydroxylase (24), a protein homologous or identical to the T_3BP (Fig. 5).

Fig. 3 shows the nucleotide sequence and predicted amino acid sequence of multifunctional T_3BP. The initiator methionine was identified based on the criteria that it was the first methionine in-frame for complete translation of the protein, and based on a comparison with other homologous proteins (Fig. 5). The amino acid sequence of the mature protein partially purified from rabbit skeletal muscle begins with the sequence NH_2-Gly-Ala-Pro-Glu-Glu-Glu-Asp-Asn-Val-Leu-Val-Leu. We have assigned the glycine in this sequence as amino acid residue 1 in Fig. 4 and the codon for it as nucleotides 1–3. The luminal localization of the T_3Bp suggests that

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-1                                                                  GGCCGGGGCCGTCGCCGCGCTCTCGTCGCCTCGGCTGTCCCGGCGGCGCCAACCGA

-56 GAAGCGCCCCGCCCGCCGCCCGAGATGCTGCGCCGCGCCGTGCTGTGCCTCGCCCTGGCCGTGACGGCCGGGTGGGCG
-18                                                MetLeuArgArgAlaValLeuCysLeuAlaLeuAlaValThrAlaGlyTrpAla

  1 TGGGCCCGCGGAGGAGGAAGACAACGTCTTGGTGCTGAAGAGCAGCAACTTCGCCGAGGAGCTGGCGGCCCACAAGCAC
  1 GlyAlaProGluGluGluGluAspAsnValLeuValLeuLysSerSerAsnPheAlaGluArgLeuAlaAlaHisLysHis

 89 CTGCTGGTGGAGTTCTATGCCCCTTGGTGCGGCCACTGCAAAGCTCTGGCGCCTGAGTACGCCAAGGCGGCCGGGAAG
 27 LeuLeuValGluPheTyrAlaProTrpCysGlyHisCysLysAlaLeuAlaProGluTyrAlaLysAlaAlaGlyLys

176 CTGAAGGCAGAGGGCTCCGACATCCGGCTGGCCAAGGTGGACGCCACCGAGGAGTCGGACCTGGCCCAGCAGTACGGC
 54 LeuLysAlaGluGlySerAspIleArgLeuAlaLysValAspAlaThrGluGluSerAspLeuAlaGlnGlnTyrGly

263 GTCCGCGGCTACCCCACGATCAAGTTCTTCAAGAATGGGGACACCGCGTCCCCCAAGGAGTACACAGCTGGCAGGGAG
 79 ValArgGlyTyrProThrIleLysPhePheLysAsnGlyAspThrAlaSerProLysGluTyrThrAlaGlyArgGlu

350 GCCGACGACATTGTGAACTGGCTGAAGAAGCCCACGGGCCCGGCTGCCACCACCCTGGCCGACAGCGCGGCCGCGGAG
105 AlaAspAspIleValAsnTrpLeuLysLysArgThrGlyProAlaAlaThrThrLeuAlaAspSerAlaAlaAlaGlu

437 TCGCTGGTGGAGTCCAGCGAGGTGGCCGTCATCGGCTTCTTCAAGGACGTGGAGTCCGACGCGGCCAAGCAGTTCCTG
131 SerLeuValGluSerSerGluValAlaValIleGlyPhePheLysAspValGluSerAspAlaAlaLysGlnPheLeu

524 CTGGCCGCGGAGGCCACCGACGACATCCCGTTCGGGCTCACGGCGAGCAGCGACGTGTTCTCCAGGTACCAGGTGCAC
157 LeuAlaAlaGluAlaThrAspAspIleProPheGlyLeuThrAlaSerSerAspValPheSerArgTyrGlnValHis

611 CAGGACGGCGTGGTGCTCTTCAAGAAGTTTGACGAGGGACGGAACAACTTCGAGGGGGAGGTCACCAAGGAGAAGCTG
183 GlnAspGlyValValLeuPheLysLysPheAspGluGlyArgAsnAsnPheGluGlyGluValThrLysGluLysLeu

698 CTGGACTTCATCAAGCACAACCAGCTGCCGCTGGTCATCGAGTTCACCGAGCAGACGGCCCCGAAAATCTTCGGAGGT
209 LeuAspPheIleLysHisAsnGlnLeuProLeuValIleGluPheThrGluGlnThrAlaProLysIlePheGlyGly

785 GAGATCAAGACACACATCCTGCTGTTCCTGCCCAGGAGCGCGGCCGACCACGACGGCAAGCTGAGCGGCTTCAAGCAG
235 GluIleLysThrHisIleLeuLeuPheLeuProArgSerAlaAlaAspHisAspGlyLysLeuSerGlyPheLysGln

872 GCGGCCGAGGGCTTCAAGGGCAAGATCCTGTTCATCTTCATCGACAGCGACCACGCCGACAACCAGCGCATCCTCGAG
261 AlaAlaGluGlyPheLysGlyLysIleLeuPheIlePheIleAspSerAspHisAlaAspAsnGlnArgIleLeuGlu

959 TTCTTCGGGCTGAAGAAGGAGGAGTGCCCGGCCGTGCGGCTCATCACGCTGGAGGAGGAGATGACCAAGTACAAGCCC
287 PhePheGlyLeuLysLysGluGluCysProAlaValArgLeuIleThrLeuGluGluGluMetThrLysTyrLysPro

1024 GAGTCGGACGAGCTCACGGCCGAGGGCATCACCGAGTTCTGCCAGCGCTTCCTGGAGGGCAAGATCAAGCCGCACCTG
313 GluSerAspGluLeuThrAlaGluGlyIleThrGluPheCysGlnArgPheLeuGluGlyLysIleLysProHisLeu

1133 ATGAGCCAGGAGCTCCCCGAGGACTGGGACCGGCAGCCGGTCAAGGTGCTGGTGGGGAAGAACTTCGAGGAGGTGGCC
339 MetSerGlnGluLeuProGluAspTrpAspArgGlnProValLysValLeuValGlyLysAsnPheGluGluValAla

1220 TTTGACGAGAAGAAGAACGTCTTCGTGGAGTTCTACGCGCCCTGGTGCGGCCACTGCAAGCAGCTGGCTCCCATCTGG
365 PheAspGluLysLysAsnValPheValGluPheTyrAlaProTrpCysGlyHisCysLysGlnLeuAlaProIleTrp

1307 GACAAGCTGGGCGAGACGTACAAGGAGCACCAGGACATCGTCATCGCCAAGATGGACTCCACGGCCAACGAGGTGGAG
391 AspLysLeuGlyGluThrTyrLysGluHisGlnAspIleValIleAlaLysMetAspSerThrAlaAsnGluValGlu

1394 GCGGTGAAAGTGCACAGCTTCCCAACGCTCAAGTTCTTCCCGGCCGGCCCCGGCAGGACGGTCATCGATTACAACGGA
417 AlaValLysValHisSerPheProThrLeuLysPhePheProAlaGlyProGlyArgThrValIleAspTyrAsnGly

1481 GAGCGGACGCTGGACGGCTTTAAGAAGTTCCTGGAAAGCGGCGGTCAGGACGGAGCAGGAGACGAGGACGGTCTGGAG
443 GluArgThrLeuAspGlyPheLysLysPheLeuGluSerGlyGlyGlnAspGlyAlaGlyAspGluAspGlyLeuGlu

1568 GACCTAGAAGAGGCCGAGGAGCCGGACCTGGAAGAAGACGACGACCAGAAAGCCGTGAGAGACGAGCTGTGAGGCCGC
469 AspLeuGluGluAlaGluGluProAspLeuGluGluAspAspAspGlnLysAlaValArgAspGluLeu

1655 ACGGAGCCGCCGGCAGGCCCTGACCACGACGCCCCGGAGCCCTGCACGGGCAGCACCGCGACCGGGGCGCCCTCCGGG
1742 CAGCCGCGCACCTGACGCCCGCGCGCCAGAGCCGCCGCCTCCTGTGCTTTTCAGTTTCTGGAAAGGAATTTATCTCCA
1829 GGCCAGCCCGTCTTGGCGGGCAGTTTTTTAAGCCGTGATGTACTTTTTTGTACACGTGTGTTGTCCGGAGGCCACACA
1916 GATGTCCGGATCTCACACTGGAGAGGTCGCGGGGTCACGGCGAATACTCCACCCGTGGGATCTGGAGACGTTTAGGAC
2003 GTCAGGGCATTGTCGCGCCGCAGCTGGCCTCCCTGGAGACGCCTGGCCGTGCGGGCGGGTGGAGCCAGCTGACACAGT
2090 CCCTGCCACCTGGGCCGGCGGAGCCAGGACGGAACCGGCCCTTGCCATTTTTTTGGTTAGACGGAGACTGTCGGAGGAG
2177 GAGGGGACTGTGGCTGCCAGCTGCGGGGCCCAGGACAGGCCTGGGCAGCCCCCTCCCTCCTCCAGCCAGGGCTCCCGT
2264 CGCCGGCGCATTGCTCTGTGGCCGCTGGACTCTCCCATGTGGCCTATGCAAGGCGGAAGCAGGACCCCAATTCCCCCG
2351 TTTTTTTTTGAGTTTTAAGCTTTGGCTTGTAGTTCTCTGGCGAATAATTTTGTTATGAAATTATCAAAGTTTAGGGCT
2438 AAGCATAGTGGGGTATCTAATCCCAGTTTGGGCCTTAGCTATCGTGAGTTCGAAGAGTATAAAATCACTTTCGTTGTT
2525 TATTTTTGTTTGCAACTATTACTTTCTACAGCTTAGTTGTTATTTGATTTAATTGTTGTTATTCTAATCACGCTTTAC
2612 GCCGGATATTTATTAATTTGGGTTAATCGTATGACGCGGTGGCTGGCACGAGATTTACCAACCCTAAAGTAGCATAAC
2699 TTAGTCAAACTTACGTTCATTGCTAAATATTTATCACTGCTGTATCCCTTGGGGGTGTGGTTAAGCAAGGCATCTTGA
2786 GCTACTGATTAGTGTGCTTAATGTCCGCTCCTCTTGATCTAGC
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FIG. 3. **Nucleotide and predicted amino acid sequences of rabbit slow-twitch skeletal muscle T_3BP.** Amino acid residues are *numbered negatively* within the 5′-untranslated region with amino acid residue 1 corresponding to the first residue of the protein. Amino acid sequence obtained by protein sequencing is *underlined*. Two repeat sequences corresponding to the predicted active sites of PDI (22, 50) are *double underlined*.

it should be made with a signal sequence that would allow it to enter the sarcoplasmic reticulum. In this respect, the NH_2-terminal sequence Met-Leu-Arg-Arg-Ala-Val-Leu-Cys-Leu-Ala-Leu-Ala-Val-Thr-Ala-Gly-Trp-Ala- upstream of the glycine between positions −18 and −1, is a typical signal sequence (49). The cleavage point between Ala and Gly is typical since a small amino acid such as Gly or Ala precedes the cleavage site in most signal sequences (49). From these observations we conclude that the initiator methionine is at the position defined in Fig. 3, that the T_3BP is made with a signal sequence and that the signal sequence is 18 amino acids in length. The two proposed active sites of PDI (22, 50), the Trp-Cys-Gly-His-Cys-Lys sequences, are found at residues 54–56 and 398–401 (Figs. 3 and 5, *double underlined*). These two sequences are located in a highly conserved region when compared to amino acid sequence of rat PDI as well as to human liver T_3BP, human hepatoma β-subunit of prolyl 4-hydroxylase, and hen oviduct glycosylation site binding protein (Fig. 5).

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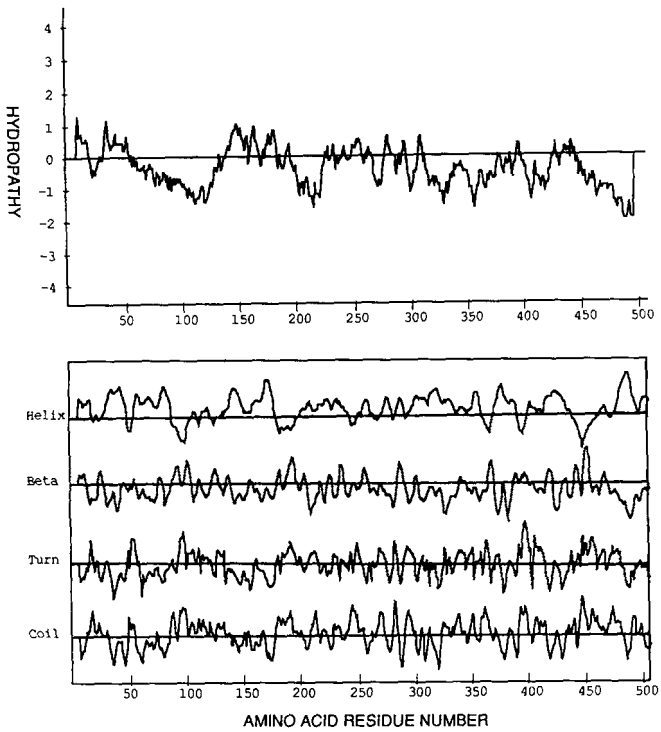

FIG. 4. **Secondary structure predictions for T_3BP.** The hydropathic character of the protein was evaluated using the procedure of Kyte and Doolittle (42) with a window of 21 amino acids (*top*). Probabilities of α helix, β sheet, β turn, and random coil structures (43, 44) in T_3BP are shown (*bottom*).

The protein has a calculated molecular weight of 56,681 and contains 95 acidic and 61 basic amino acid residues. The COOH terminus of the protein is highly enriched in acidic residues with 17 of the last 29 amino acids being negatively charged. The last 4 residues of the protein are Arg-Asp-Glu-Leu-COOH. This COOH-terminal sequence was the same in the initial partial length cDNA clones (clones 1 and 2, data not shown).

Protein Structure Analysis—The secondary structure predictions for T_3BP are shown in Fig. 4. Analysis of hydropathy suggests that there are no long hydrophobic regions in agreement with the current hypothesis that the T_3BP is a lumenal membrane protein. Two relatively hydrophilic regions occur, the first between residues 75 and 135 and the second one at the COOH-terminus of the protein (last 55 residues). This first hydrophilic region is followed by a helix-turn-helix motif (residues 136–175). This is followed by several regions predicted to form a β turn and α helix dispersed over the length of the protein. The general profile of hydropathy is similar to that of two other peripheral Ca^{2+} binding membrane proteins present in sarcoplasmic reticulum, calsequestrin (4), and calreticulin (9), especially at the COOH-terminus. This homology of the COOH-terminal amino acid sequence of T_3BP and calreticulin (Fig. 1) may be a reason for the cross-reactivity of our anti-calreticulin antibody with the COOH-terminal end of the T_3BP.

Homology to Other Proteins—A comparison of amino acid sequence of the skeletal muscle form of the 55-kDa multifunctional T_3BP (this work) with human liver T_3BP (23), PDI (21), β-subunit of prolyl 4-hydroxylase (24), and glycosylation site binding protein (16) (Fig. 5) shows that these proteins are closely related but significantly different. Some regions of the protein were identical such as amino acids residues 111–141 and 235–263. Other regions varied greatly from one protein to another particularly within the NH_2-terminal signal sequence. In a number of cases the substitutions are quite conservative Asp for Glu or Lys for Arg, while in other cases the substitutions seem much more significant. A noticeable change is in the last four amino acid residues which are RDEL in rabbit muscle T_3BP and are KDEL in all other cases. In many instances rabbit skeletal muscle T_3BP is the only protein of the group which differs from all the others particularly residues 264–269, 275, 278, 417–420, and 451–458.

Northern Analysis and Tissue Distribution of T_3BP mRNA—cDNA fragments encoding either NH_2-terminal or COOH-terminal regions of the T_3BP hybridized to the same RNA species in Northern blots from a variety of tissues. Fig. 6 shows Northern blot analysis with probes from the 5′ end of the clone. The cDNA hybridized to mRNAs of 2.7 kb in all the tissues tested; liver, kidney, brain, cardiac muscle, and fast- and slow-twitch rabbit skeletal muscle. In all cases, the probe hybridized to a much greater extent with the poly $(A)^+$ RNA in comparison with total RNA, suggesting that the binding was specific to a mRNA species.

Isolated Sarcoplasmic Reticulum Vesicles Contain a 55-kDa Multifunctional T_3BP—To confirm that the 55-kDa multifunctional T_3BP protein is present in isolated sarcoplasmic reticulum a monoclonal antibody against purified T_3BP from rat liver endoplasmic reticulum was tested for its immunoreactivity with rat skeletal muscle sarcoplasmic reticulum proteins (Fig. 7). Rat liver endoplasmic reticulum membranes were used as a positive control (Fig. 7, *lane 1*). Anti-liver endoplasmic reticulum T_3BP monoclonal antibody (26) recognized a 55-kDa protein band in both liver endoplasmic reticulum and skeletal muscle sarcoplasmic reticulum membranes (Fig. 7). Thus, a 55-kDa multifunctional thyroid hormone binding protein appears to be located in the sarcoplasmic reticulum similar to liver endoplasmic reticulum.

DISCUSSION

We have cloned and sequenced the 55-kDa multifunctional T_3BP of a slow-twitch rabbit skeletal muscle. The clone encodes a polypeptide of 509 amino acid residues and the molecular weight of the mature protein is 56,681. The COOH-terminus of the protein is highly enriched in acidic residues with 17 of the last 29 amino acids being negatively charged. The protein is synthesized with an 18-amino acid NH_2-terminal signal sequence.

The rabbit slow-twitch muscle cDNA clone encoding T_3BP is clearly related to a number of other proteins. These include; PDI (21), glycosylation site binding protein (16), the β-subunit of prolyl 4-hydroxylase (24), and liver T_3BP (23). The identity of the skeletal muscle T_3BP clone with PDI is further supported by the presence of two repeated sequences Trp-Cys-Gly-His-Cys-Lys, both of which are in highly conserved portions of the molecule (residues 54–56, 398–401). These sequences are proposed to be in the active sites of PDI (22, 50). This homology is further extended to other proteins. Significant sequence homologies also exist between PDI and glutathione-insulin transhydrogenase (51) and between PDI and form I phosphoinositide-specific phospholipase C (52) and a developmentally regulated trypanosome gene BS2 (53). PDI also has significant homology to the E_2 domain of the human estrogen receptor (54). Recently, the 58-kDa subunit of triglyceride transfer protein was identified as PDI (55). A rat cDNA clone was isolated in a study on iodothyronine-5′-monodeiodinase, which differed in only two amino acids from rat PDI (56). The identity of the iodothyronine-5′-monodeiodinase to PDI, however, has been questioned (57). Recently Mazzarella *et al.* (58) have cloned another luminal

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                  10         20         30         40         50         60         70         80         90
Muscle T3   MLRRAVLCLA LAVTAGWAGA PEEEDNVLVL KSSNFAEALA AHKHLLVEFY APWCGHCKAL APEYAKAAGK LKAEGSDIRL AKVDATEESD
Liver T3    .....L.... V.ALV.AKA- .......... RK........ ...Y...... .......... .......... ......E... ..........
PDI         ..S..L.... ..ARV.-.D. A......... .K......P. ..NY...... .......... .....-..A. ......E... ..........
Hydroxylase .....L...P W.XALVR.D. .....H.... RK........ ...YPP...H .......... .......... ......E... ..........
GSBP        ...-.-.... ..WRVRVGAD A..Q.....A .K...L-P.. ..SY.A.... .......... ..D....G.. ......E.KA ...E......

                 100        110        120        130        140        150        160        170        180
Muscle T3   LAQQYGVRGY PTIKFFKNGD TASPKEYTAG READDIVNWL KKRTGPAATT LADSAAAESL VESSEVAVIG FFKDVESDAA KQFLLAAEAT
Liver T3    .......... ......R..- .......... .......... .......... .R.G...... .G........ ........S. ....Q....I
PDI         .......... .......... .......... .......... .......... .S.T...... .D....T... ....AG..S. .........V
Hydroxylase .......... ......R... .......... .......... .......... .P.G...... .......... ........S. ....Q....I
GSBP        ........A. .......... .......... .......... .......... .S.T...... .....IT.IIG....PG..S. R......D.V

                 190        200        210        220        230        240        250        260        270
Muscle T3   DDIPFGLTAS SDVFSRYQVH QDGVVLFKKF DEGRNNFEGE VTKEKLLDFI KHNQLPLVIE FTEQTAPKIF GGEIKTHILL FLPRSAADHD
Liver T3    ..V...I.SN .....K..LD K.......... .......... .......... .......... .......... .......... ...K.VS.Y.
PDI         ......I.SN .....K..LD K.......... .......... I......... .......... .......... .......... ...K.VS.Y.
Hydroxylase ......I.SN .....K..LD K.......... .......... ....N..... .......... .......... .......... ...K.VS.Y.
GSBP        V..V...INSN ...Y.K..MD K.A....... A......... I......... ...N...... .......... .......... ...K.VS.Y.

                 280        290        300        310        320        330        340        350        360
Muscle T3   GKLSGFKQAA EGFKGKILFI FIDSDHADNQ RILEFFGLKK EECPAVRLIT LEEEMTKYKP ESDELTAEGI TEFCQRFLEG KIKPHLMSQE
Liver T3    ....N..T.. .S........ ......T... .......... .......... ........-. ..E.....R. ....H..... ..........
PDI         ....N..K.. .......... ......T... .......... .......... ........-. ........K. .Q..HH.... ..........
Hydroxylase ....N..T.. .S........ ......T... .......... .......... .......... ..E.....R. ....H..... ..........
GSBP        ....NL.K.. D........V ......T... .......... .......... .D..L..... .TE.....KL .Q..HH.... ........N.

                 370        380        390        400        410        420        430        440        450
Muscle T3   LPEDWDRQPV KVLVGKNFEE VAFDEKKNVF VEFYAPWCGH CKQLAPIWDK LGETYKEHQD IVIAKMDSTA NEVEAVKVHS FPTLKFFPAG
Liver T3    RAG...K... ..P......D .......... .......... .......... ......D.EN .......... .......... .........S
PDI         ....S.K... .......... .......... .......... .......... ......D.EN .......... .......... .........S
Hydroxylase ......K... .........D C......... .......... ..............D.EN .......... .........G ....G....S
GSBP        P......K... .......Y.. .......... I......... ......M..R ..A-..DDEN ......E... .....I.... .........S

                 460        470        480        490        500        509
Muscle T3   PGRTVIVNNG ERTLDGFKKF LESGGQDGAG DEDGLEDLEE AEEPDLEEDD DQKAVRDEL
Liver T3    AD....DY.. .......... .......... .D.-...... .....M.... .....K...
PDI         AD....DY.. .......... .......... .N.D.-.... .L...M.... .....K...
Hydroxylase AD....DY.. .......... .......... .V.D...... .....M.... .....K...
GSBP        AE....DY.. ......Y... ........Y. NN.D-DD... .L.S.M...E ....MK...
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FIG. 5. **Comparison of deduced amino acid sequence of rabbit slow-twitch muscle T_3BP (*muscle T_3*) with human liver T_3BP (*liver T_3*) (23), rat liver PDI (21), human hepatoma β subunit of prolyl 4-hydroxylase (*hydroxylase*) (24), hen oviduct glycosylation site binding protein (*GSBP*) (16).** Regions of identity are noted by a *period* and differing amino acids are shown. Two repeat sequences corresponding to the predicted active sites of PDI (22, 50) are *double underlined*.

endoplasmic reticulum protein ERp72 which shares some sequence identity with PDI including the active site of PDI. Our clone is highly homologous but not identical to the above described group of proteins. The differences are not simply species related since for example, chicken oviduct glycosylation site binding protein appears to be more closely related to the rat and human clones than to the rabbit skeletal muscle form of the protein (Fig. 5, compare residues 187–200, 339–345, 417–420, 450–458). This clone may therefore encode for a homologous protein with either different function or one that is regulated in a different manner.

Whether or not this group of proteins with different functions are indeed all the same protein or are different but homologous proteins, has not yet been resolved. This multifunctional protein may serve as a common subunit of many larger protein complexes involved in a co-translational modification of proteins. The thyroid hormone binding to this protein may be the mechanism by which the hormone regulates the translation and/or modification of newly synthesized proteins. Based on the results of this work, on described sequence homologies and on our earlier observations that T_3 binds to the solubilized sarcoplasmic reticulum membrane (14) we conclude that sarcoplasmic reticulum membrane contains the 55-kDa multifunctional T_3BP and that the cDNA clone isolated in this work may encode the skeletal muscle form of this unique protein. This conclusion is also supported by our immunoblot analysis of sarcoplasmic reticulum protein with anti-T_3BP monoclonal antibody (Fig. 7).

It was unexpected that the initial cDNA clone (clone 1) encoding the COOH-terminal amino acid sequence of T_3BP was isolated from the λgt11 expression library using a polyclonal antibody against calreticulin (9). The reason for the cross-reactivity of anti-calreticulin antibody with T_3BP is probably due to similarity in the two proteins amino acid sequences. Our goat anti-calreticulin antibody was earlier shown to react with the COOH-terminal sequence of calreticulin (9) and both proteins contain similar although not identical COOH-terminal amino acid sequences (Fig. 1) with similar hydrophilic predicted protein structures at their COOH termini. Our polyclonal antibody generated against calreticulin is thus likely reacting against this specific COOH-terminal sequences in T_3BP. The reactivity of this antibody with T_3BP, however, is much weaker than with calreticulin and the antibody did not cross-react with purified T_3BP in Western blots. The reason for this discrepancy requires further studies.

One of the most interesting features about the sequence of this T_3BP cDNA clone is the tetrapeptide at its COOH terminus. This sequence Arg-Asp-Glu-Leu-COOH is similar to the KDEL sequence Munro and Pelham (27) have shown may be responsible for retention of newly synthesized proteins within the lumen of the endoplasmic reticulum. A number of

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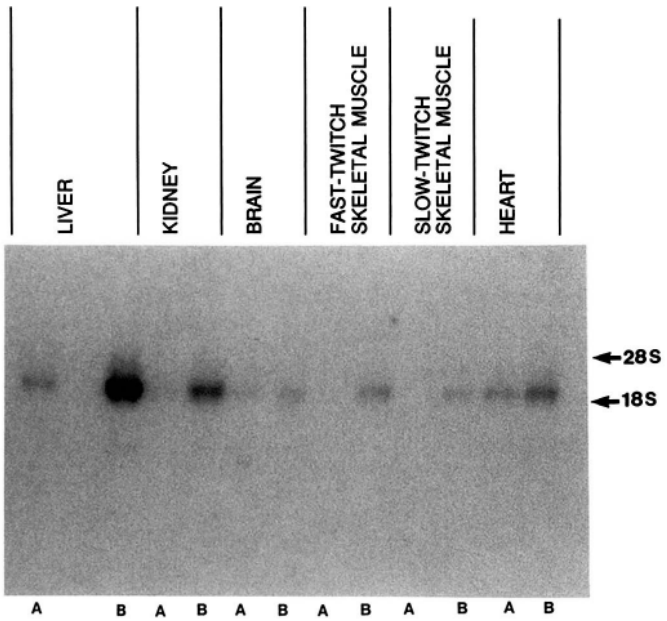

FIG. 6. **Northern blot analysis of RNA from rabbit tissues.** Total (*A*) and poly(A)$^+$ RNAs (*B*) from rabbit liver, kidney, brain, fast-twitch skeletal muscle (psoas), slow-twitch skeletal muscle (soleus), and cardiac muscle were isolated and fractionated on formaldehyde-agarose gels as described under "Experimental Procedures." The position of the 28 S and 18 S ribosomal subunits are indicated. The size of the mRNA hybridizing to T_3BP cDNA was estimated to be 2.7 kb.

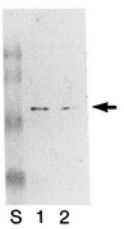

FIG. 7. **Identification of the 55-kDa multifunctional T_3BP in sarcoplasmic reticulum membrane.** Endoplasmic reticulum and sarcoplasmic reticulum membrane fractions were prepared as described under "Experimental Procedures." Membrane proteins were separated on SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and immunoreacted with anti-rat liver endoplasmic reticulum T_3BP monoclonal antibody (26). *Lane 1,* rat liver endoplasmic reticulum; *lane 2,* rat skeletal muscle sarcoplasmic reticulum. Protein amounts were 35 μg/lane. *S,* prestained molecular weight standards. *Arrow,* location of a 55-kDa immunoreactive protein band.

variations of this sequence have been reported (28) including the sequence KEEL-COOH for the protein ERp72 (58) and HDEL-COOH in yeast (28). The sequence of the rabbit muscle form of this protein appears to suggest that a substitution of a Lys residue to an Arg residue is sufficient to retain a functional signal. It appears as though a retention of the positive charge at this position may be crucial and sufficient to maintain an active signal. This retention sequence cannot be muscle-specific since the skeletal muscle form of calreticulin terminates with a KDEL signal (9). Another interesting observation about the COOH-terminal sequence of the KDEL proteins is the predominance of negatively charged amino acids. Both calreticulin and T_3BP have more acidic residues than would be expected to be present by random chance. The function of these acidic residues is unknown. These highly charged amino acid sequences upstream of the K(R)DEL COOH-terminal sequence together with the K(R)DEL sequence may form a retention signal for the endoplasmic reticulum proteins (12, 28). Acidic COOH-terminal amino acid sequences present in T_3BP may be involved in Ca^{2+} binding similar to that proposed for calsequestrin and calreticulin (4, 9).

A number of other sarcoplasmic reticulum peripheral membrane proteins have recently been cloned including calsequestrin (4, 5), 160-kDa sarcolumenin (6), and 53-kDa glycoproteins (7), the 165-kDa histidine-rich, Ca^{2+} and low density lipoprotein binding protein (HCP) (8), and calreticulin (9). Only calreticulin (9) and T_3BP contain the K(R)DEL retention sequence allowing them to be salvaged by the mechanism proposed by Munro and Pelham (27). The mechanism responsible for maintaining the intracellular location of proteins without this sequence is not yet known (12), but may involve a specific receptor as suggested for calsequestrin (59). Protein-protein interactions and/or protein aggregation (self-association) may play an additional role in retaining some sarcoplasmic and endoplasmic reticulum peripheral membrane proteins. In this respect the sequence Ser-Glu-Glu, suggested to play an important role in protein-protein interaction among several nuclear proteins (60), and it occurs in sarcolumenin (6), 53-kDa glycoprotein (7), calsequestrin (4), and 165-kDa HCP (8). It should be noted that in addition to calsequestrin, which is self associating in the presence of Ca^{2+}, the sequence Ser-Glu-Glu occurs in troponin C and calmodulin, which clearly interact with other proteins in the presence of Ca^{2+}. Calreticulin does not contain the Ser-Glu-Glu sequence (9) and does not form a part of a larger protein complex. The sequence Ser-Glu-Glu occurs once in human liver T_3BP (23) and in the β subunit of prolyl 4-hydroxylase (24) and a modified version Ser-Asp-Glu occurs in the same position in PDI (22) and skeletal muscle T_3BP (residues 332–334). Similar to that postulated for other proteins, this sequence may play a critical role in the association of this multifunctional subunit with other proteins. It will be interesting to look at the possible role of this and other sequence in the interactions of these multifunctional proteins.

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Molecular cloning of cDNA encoding a 55-kDa multifunctional thyroid hormone binding protein of skeletal muscle sarcoplasmic reticulum.

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