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cDNA cloning and sequencing of phospholamban from pig stomach smooth muscle

Hilde VERBOOMEN, Frank WUYTACK,* Jan A. EGGERMONT, Sabine DE JAEGERE, Ludwig MISSIAEN, Luc RAEYMAEKERS and Rik CASTEELS

Laboratorium voor Fysiologie, Katholieke Universiteit Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

Phospholamban cDNA from pig stomach smooth muscle was cloned and sequenced. The 737-nucleotide-residue cDNA contained an open reading frame of 156 nucleotide residues encoding a peptide of 52 amino acid residues (M_r 6080). This peptide shares 100% sequence identity with dog cardiac-muscle phospholamban. It differs from rabbit cardiac-muscle and slow-twitch skeletal-muscle phospholamban only at position 2, which is a glutamic acid residue in rabbit phospholamban, but an aspartic acid residue in the pig smooth-muscle protein. Northern-blot analysis reveals the presence of several phospholamban mRNAs in smooth muscle, but a 900-nucleotide-residue and a 2800-nucleotide-residue transcript predominate.

INTRODUCTION

Phospholamban (PLB) is a small protein (M_r 6000) with a strong tendency to form homopentameric complexes [1]. The protein is thought to regulate in a phosphorylation-dependent manner the Ca^{2+} content in the sarcoplasmic reticulum/endoplasmic reticulum of different muscle types. PLB was first described in the sarcoplasmic reticulum of cardiac muscle and slow-twitch skeletal muscle [2], but has also been demonstrated in smooth muscle [3]. Cyclic AMP-dependent protein kinase [4], cyclic GMP-dependent protein kinase [5], Ca^{2+} + calmodulin-dependent protein kinase [6] and protein kinase C [7] each phosphorylates PLB. Phosphorylation of PLB causes a conformational change of the protein that might be responsible for the observed changes in activities of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase and of Ca^{2+} transport into sarcoplasmic-reticulum vesicles. The mode of interaction of PLB with the Ca^{2+}-transport ATPase remains, however, to be elucidated. Recently the primary structure of dog cardiac-muscle PLB was elucidated both by peptide sequencing [8] and by cDNA sequencing [9,10]. cDNA sequencing also yielded the nucleotide sequence and the derived amino acid sequence of the rabbit cardiac-muscle and slow-twitch skeletal-muscle PLB [11]. We now report the cloning and sequencing of PLB from a cDNA library of pig stomach smooth muscle.

MATERIALS AND METHODS

RNA isolation and cDNA synthesis

Total RNA was extracted from the smooth-muscle layer of a 2-day-old piglet according to the procedure of Chirgwin *et al.* [12]. A poly(A)-enriched RNA fraction was isolated via oligo(dT)–cellulose chromatography [13]. Polyadenylated RNA (5 μg) was oligo(dT)-primed and reversely transcribed with avian-myeloblastosis-virus reverse transcriptase. After use of the Gubler & Hoffman method [14] for second-strand synthesis and standard processing of the cDNA [15] it was ligated into *Eco*RI-cut and dephosphorylated bacteriophage λ gt11 arms (Promega, Madison, WI, U.S.A.). After plating of the library in *Escherichia coli* Y1090, about 10^6 independent clones were obtained from 190 ng of cDNA. The cDNA library was amplified and stored at 4 °C.

Screening procedures

Screening was done with an oligonucleotide probe of the following sequence: 5′-TTTTGACGTGCTTGTTG-AGGCATTTCAATGGTTGA-3′. This is the inverse complement of an identical sequence that is represented both in the cDNA sequence of dog cardiac-muscle PLB [9,10] and in rabbit cardiac-muscle or slow-twitch skeletal-muscle PLB [11]. The probe was 5′-end-labelled to a specific radioactivity of 10^8 c.p.m./μg of DNA by using a bacteriophage-T4-polynucleotide kinase-based method [16]. Samples of the cDNA library were plated and transferred to nylon filters (Hybond-N; Amersham International, Amersham, Bucks., U.K.) and the filters were prehybridized during 4 h at 42 °C in a mixture containing 5 × SSPE, 0.1% SDS, 5 × Denhardt's solution, 0.05 mg of denaturated salmon sperm DNA/ml and 0.2 mg of tRNA/ml (1 × SSPE is 0.15 M-NaCl/0.001 M-EDTA/0.01 M-sodium phosphate buffer, pH 7.4 and 1 × Denhardt's solution contains 0.2 g of polyvinylpyrrolidone, 0.2 g of bovine serum albumin and 0.2 g of Ficoll 400 per l of water). Overnight hybridization was done at 42 °C with the same mixture containing the probe at a concentration of approx. 10^6 c.p.m./ml. Filters were washed with a final stringency of 6 × SSC/0.1% SDS at 50 °C (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate buffer, pH 7.0).

Subcloning and sequencing procedures

The cDNA inserts of clones PLB-7 and PLB-8 were excised from the λ bacteriophages by *Eco*RI digestion and subcloned into the pGEM-7 Zf(+) vector (Pro-

Abbreviations used: PLB, phospholamban; nt, nucleotide residue(s).

* To whom correspondence should be addressed.

These nucleotide sequence data have been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X15075.

mega). Dideoxy sequencing was done on the plasmids by the Sequenase method with the commercially available universal and reverse sequencing primers (United States Biochemical Co., Cleveland, OH, U.S.A.). Two additional primers (17-mers), P_1 (5′-ACTTCTTCTGCTGAGGA-3′) and P_2 (5′-TTGCATCATCGTGATGC-3′), were synthesized and used to extend the sequences further.

Northern blotting

RNA samples were denatured before electrophoresis by glyoxalation [16]. Gels of 1% agarose in 10 mM-sodium phosphate buffer, pH 7.0, were run at 3 V/cm for 3 h at room temperature with continuous buffer recirculation. A 0.24–9 kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) was used for sizing. After transfer this part of the blot was stained separately with Methylene Blue [16].

After electrophoresis, RNA was capillary-transferred for 3 h on to a positively charged Hybond-N+ nylon membrane (Amersham International). The transfer solvent was 50 mM-NaOH. Prehybridization was conducted for 3 h at 50 °C in a solution containing 50% (v/v) formamide, 5 × SSPE, 5 × Denhardt's solution, 0.05 mg of denaturated salmon sperm DNA/ml and 0.2 mg of tRNA/ml. Hybridization was done for 12 h in the same solution with the RNA probe added (10^9 c.p.m./μg of RNA; 10^7 c.p.m./ml of hybridization mixture). Blots were rinsed in 2 × SSPE and then treated for 30 min at 37 °C with 10 μg of RNAase A/ml in 2 × SSPE. It was successively washed for each condition twice for 15 min, first in 1 × SSPE / 0.1% SDS at 20 °C, then in 0.1 × SSPE/0.1% SDS at 65 °C and finally in 0.1 × SSPE at 20 °C.

Preparation of the RNA probe

The PLB-8 insert in pGEM-7 Zf(+) was linearized with *Hin*dIII. By using the Promega Riboprobe Gemini system in conjunction with the bacteriophage T7 RNA polymerase and CTP at 40 mCi/ml and 250 μCi/reaction mixture, a high-specific-radioactivity (10^9 c.p.m./μg of RNA) antisense RNA probe was generated.

RESULTS AND DISCUSSION

A synthetic DNA oligomer based on a perfectly identical sequence in dog cardiac-muscle [9,10] and in rabbit cardiac-muscle and slow-twitch skeletal-muscle PLB cDNA [11] was used as a probe to screen an amplified pig antral smooth-muscle cDNA library in bacteriophage λ gt11. A total of 2×10^5 plaques were screened, which resulted in two positive clones PLB-7 and PLB-8 that were about 800 nt long. The cDNA inserts were subcloned into pGEM-7 Zf(+) and sequence analysis was performed on double-stranded plasmid DNA. The sequencing strategy is indicated in Fig. 1. PLB-7 and PLB-8 cDNA sequences were found to be identical except for their 3′-termini: PLB-7 contained three additional nucleotide residues at the 3′-end followed by a poly(A) tail. An open reading frame of 156 nucleotide residues (nt 1 to nt 156 in Fig. 2) encoded a peptide of 52 amino acid residues (M_r 6080). This peptide shares 100% sequence identity with dog cardiac-muscle PLB [9, 10] and it differs from the rabbit cardiac-muscle and slow-twitch skeletal-muscle PLB only at position 2 [11]: the second amino acid residue of the rabbit cardiac-muscle/slow-twitch skeletal-muscle PLB is glutamic acid whereas it is aspartic acid in the pig smooth-muscle clones (see Fig. 2). We therefore conclude that the PLB-7 and PLB-8 cDNA clones correspond to smooth-muscle PLB transcripts. The high degree of sequence identity between cardiac-muscle and smooth-muscle PLB is in agreement with previous observations that had already demonstrated a high degree of similarity between the properties of cardiac-muscle and smooth-muscle PLB [3,5].

Northern-blot analysis reveals the presence of several mRNAs of which a short transcript of 900 nt and a long one of 2800 nt are the most predominant (see Fig. 3). The

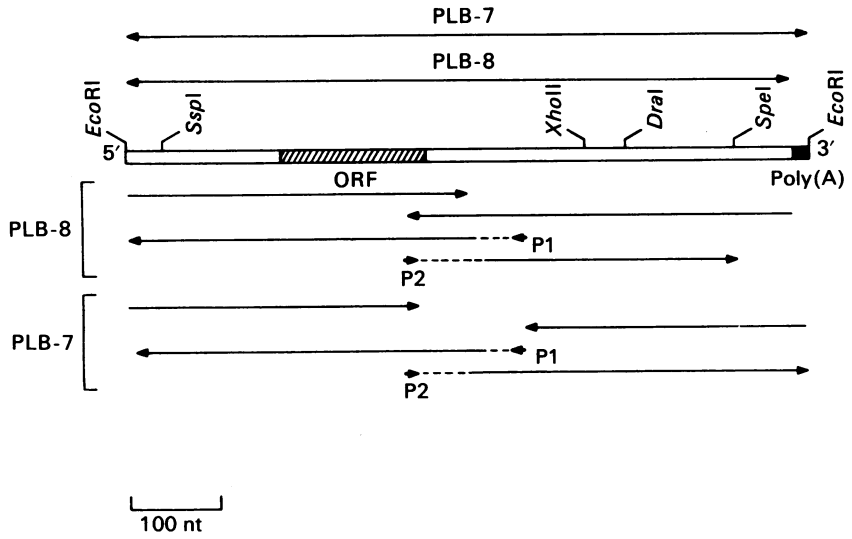

Fig. 1. Sequencing strategy for cDNA clones PLB-7 and PLB-8

Plasmid sequencing of the two clones was performed in both directions. The complete sequence was obtained by using two additional 17-mer oligonucleotide primers (P_1 and P_2), both deduced from already known nucleotide sequences. Poly(A) indicates the poly(A) tail (17 nt long). Abbreviation: ORF, open reading frame.

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-172          CTTTCCAGCTAAACACCGATAAGACTTCATACAACTCACAATATTCTATACT

-120  GTGATCATCATAAACAGCCAAGGCTGCCTAAAAGAAGACAGTGATCTCATATTTGGCTGG

 -60  TAGCTTTTTCAGCTTTCTCTTGACGGCTTAAAACTTCAGGCTTCCTGCCTTCCTGGTATC

   1  ATGGATAAAGTCCAATACCTCACTCGCTCTGCTATTAGAAGAGCTTCAACCATTGAAATG
      M  D  K  V  Q  Y  L  T  R  S  A  I  R  R  A  S  T  I  E  M   20

  61  CCTCAACAAGCACGTCAAAACCTTCAGAACCTATTTATCAATTTCTGTCTCATCTTAATA
      P  Q  Q  A  R  Q  N  L  Q  N  L  F  I  N  F  C  L  I  L  I   40

 121  TGCCTCTTGCTGATTTGCATCATCGTGATGCTTCTCTGAAGTTCTGCCACAATCTCCAGT
      C  L  L  L  L  I  C  I  I  V  M  L  L  *                     52

 181  GCTACAATTTATCACCATCAGCTTAAAATCTGTCATCCCATGAAGAAGGGAAACAATACT

 241  ATATAAGAGACCACTTCCTCAGCAGAAGAAGTTCTTTATGAAAAGGTCAAGATTTGGAAT

 301  GAAACAAATTGTTAGCAAATGTATTCATCTGCTGGATCTTGTAAACATGAAAAGGGCTTT

 361  ATTTTCAGAAACTAACTTTAAAATGACTATAAGTGTGCATAATGCAACTGTTGATTTTCT

 421  CAACAGAGCTTATAAATTTCTATCCCAAATCTTTTCTGAGGATGAACTAGGGGTTTAGTT

 481  TGGAAACTGCACTGCTAACTAGTTCACGTCATATATAAAGCATTAGCTTCACTCTTTGGG

 541  GTAAATATAATTTATACTGTACTGT-poly(A)
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Fig. 2. Nucleotide sequence and translated amino acid sequence of PLB cDNA

Nucleotide residues are numbered at the left, amino acid residues at the right.

mRNA heterogeneity is detected in smooth muscle as well as in cardiac muscle. This is in agreement with the data of Uyeda *et al.* [10], who described in dog cardiac muscle several PLB mRNAs ranging in size from 1.0 kb to 3.6 kb, 2.8 kb being a major species. Also, the rabbit PLB mRNA can be cleaved and poly(A)-tailed at several distinct sites [11]. Interestingly, several polyadenylation signals differ from the consensus sequence AATAAA. This probably also applies to the pig smooth-muscle PLB transcripts. The PLB-7 cDNA clone, which ends in a poly(A) tail, contains an AATATA sequence 23 nt upstream of the poly(A)-addition site (nt 544 to nt 549 in Fig. 2). If the AATATA sequence, which differes from the consensus polyadenylation sequence AATAAA in only one nucleotide residue, is recognized as a polyadenylation signal, a short mRNA will be generated. However, by analogy to the rabbit cardiac-muscle/slow-twitch skeletal-muscle transcripts we presume that the AATATA sequence can also remain silent, which would result in longer PLB messengers cleaved at more distally located sites.

A comparison of the pig stomach smooth-muscle PLB cDNA with the corresponding sequences of dog and rabbit cardiac-muscle PLB cDNA indicates a high degree of sequence similarity. The sequence similarity between pig and dog is slightly higher than that between pig and rabbit. PLB cDNA from pig stomach smooth muscle shows 85% nucleotide identity with the PLB cDNA from dog cardiac muscle and 82% identity with that from rabbit cardiac muscle. As expected, the sequence similarity is higher in the protein-coding region, where respectively 96% and 93% identity is observed. The high degree of sequence similarity between the cardiac cDNA (if allowance is made for the species differences) and the identical size distribution of mRNAs suggest that cardiac muscle and smooth muscle express the same PLB gene.

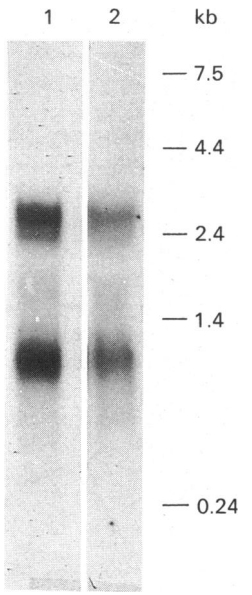

Fig. 3. Northern-blot analysis of pig cardiac-muscle and pig stomach smooth-muscle total RNA

Pig cardiac-muscle total RNA (10 μg) (lane 1) and stomach smooth-muscle total RNA (20 μg) (lane 2) were electrophoresed, capillary-blotted and hybridized with an antisense [^{32}P]RNA PLB-8 probe as indicated in the Materials and methods section. Autoradiography at −80 °C with intensifying screens lasted for 5 h and 24 h respectively for lanes 1 and 2.

In conclusion, pig antrum smooth muscle presents an mRNA encoding a protein identical with that in dog cardiac muscle. Phosphorylation of well-characterized endoplasmic-reticulum fractions from smooth muscle,

both by cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase, is accompanied by a much lower degree of stimulation of Ca^{2+} transport as compared with the situation in cardiac sarcoplasmic reticulum (L. Raeymaekers, J. A. Eggermont, M. Vrolix, F. Wuytack & R. Casteels, unpublished work). The explanation for this discrepancy should therefore not be sought in isoform diversity of phospholamban in the two tissues.

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