Purification and Complete Sequence Determination of the Major Plasma Membrane Substrate for cAMP-dependent Protein Kinase and Protein Kinase C in Myocardium*

Cathy J. Palmer, Bruce T. Scott, and Larry R. Jones

From the Kranert Institute of Cardiology and the Departments of Medicine and Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana 46202

A protein of apparent \( M_r \) = 15,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis is the major plasma membrane substrate for cAMP-dependent protein kinase (PK-A) and protein kinase C (PK-C) in several different tissues. In the work described here, we purified, cloned, and sequenced the canine cardiac sarcolemmal “15-kDa protein.” The amino terminus of the purified protein was not blocked, allowing determination of 50 consecutive residues by standard Edman degradation. Overlapping proteolytic phosphopeptides yielded 22 additional residues at the carboxyl terminus. Dideoxy sequencing of the full-length cDNA confirmed that the 15-kDa protein contains 72 amino acids, plus a 20-residue signal sequence. The mature protein has a calculated \( M_r \) = 8409. There is one hydrophobic membrane-spanning segment composed of residues 18–37. The acidic amino-terminal end (residues 1–17) of the protein is oriented extracellularly, whereas the basic carboxyl-terminal end (residues 38–72) projects into the cytoplasm. The positively charged carboxyl terminus contains the phosphorylation sites for PK-A and PK-C. In the transmembrane region, the 15-kDa protein exhibits 52% amino acid identity with the “γ” subunit of Na,K-ATPase. High stringency Northern blot analysis revealed that 15-kDa mRNA is present in heart, skeletal muscle, smooth muscle, and liver but absent from brain and kidney. We propose the name “phospholemman” for the 15-kDa protein, which denotes the protein’s location within the plasma membrane and its characteristic multisite phosphorylation.

A protein of apparent \( M_r \) = 15,000 is the major plasmalemmal substrate for PK-A\(^1\) and PK-C in several different tissues. This “15-kDa protein” was first described in cardiac sarcolemmal vesicles (1–5) and has since been identified in plasma membranes from skeletal (6, 7) and smooth (8–10) muscle, liver (11), and adrenal tumor cells (12). Stimulation of these tissues with different agonists leads to phosphorylation of the 15-kDa protein by Ca\(^{2+}\) and cAMP-dependent mechanisms (8–14). In cardiac muscle, phosphorylation of the 15-kDa protein occurs after activation of either \( \alpha \) - or \( \beta \)-adrenergic receptors, and correlates with an increase in contractility (13, 14). In spite of its prominence as a major plasma membrane phosphoprotein, the precise function of the 15-kDa protein remains undefined. No sequence information on the protein has yet been reported nor has the protein been purified.

In the work described here, we report on the purification and complete amino acid sequence of the cardiac sarcolemmal 15-kDa protein. The protein is quite small and contains a single transmembrane domain. A highly basic carboxyl-terminal tail projects into the cytoplasm, which contains several protein kinase phosphorylation sites. Knowledge of the protein structure gives some clues regarding 15-kDa protein function, which are briefly discussed.

**EXPERIMENTAL PROCEDURES**

Isolation of \( ^{32} \)P-Labeled 15-kDa Protein from Canine Cardiac Sarcolemmal Vesicles—Sarcolemmal vesicles were isolated from dog left ventricles as described previously (15). By omitting NaCl from the homogenization buffer and gradient solutions, membranes were obtained which exhibited 5-fold greater phosphorylation of the 15-kDa protein compared with our earlier study (5). Protein concentrations were determined by the method of Lowry et al. (16).

Sarcolemmal vesicles were permeabilized by freeze-thaw shock and phosphorylated by endogenous PK-C (5). Freeze-thaw-treated sarcolemmal vesicles were preincubated for 2 min at 30°C in buffer containing 75 mM Pipes-Tris (pH 6.8), 7.5 mM MgCl\(_2\), 0.75 mM EGTA, and 0.88 mM CaCl\(_2\) (1.0 mg of protein/2 ml). Phosphorylation was initiated by adding 80 \( \mu \)M \[^{32} \)P\]ATP (500 \( \mu \)Ci/mg protein) and incubating at 30°C for 2 min. Reaction mixtures were then centrifuged at 500,000 \( \times \) g for 7 min at 4°C. Pellets were solubilized in electrophoresis sample buffer (5), and SDS-PAGE was performed according to the method of Laemmli (17) using three-well 15% polyacrylamide gels (16 cm \( \times \) 18 cm \( \times \) 1.5 mm). Each well was loaded with 325 \( \mu \)g of solubilized sarcolemmal membranes containing approximately 125 pmol of \[^{32} \)P-labeled 15-kDa protein. Following electrophoresis, sample lanes were cut horizontally into 2-mm slices and analyzed for labeled 15-kDa protein by Cerenkov counting. Radioactive protein was electroeluted using a Bio-Rad Mini Protean apparatus and then concentrated using Centricon-10 Microcentrifugation (Amicon) and precipitated (18). Protein precipitates were solubilized in 70% formic acid containing 1 mg/ml CNDr and incubated in absence of light at 25°C for 16 h and then dried in a Savant Speed-Vac and solubilized in electrophoresis sample buffer. SDS-PAGE was performed, and sample lanes were cut and analyzed by Cerenkov counting as above. The 15-kDa protein was electroeluted, concentrated, and precipitated as before. Some fractions were further purified by high pressure liquid chromatography using a C\(_18\) reverse-phase column equilibrated with 0.1% trifluoroacetic acid in water and...
developed with a linear gradient of 0.1% trifluoroacetic acid in propanol-1. Column fractions were monitored by absorbance at 214 nm and by Cerenkov counting.

**Proteolytic Digestion and Purification of Phosphopeptides**—Purified and precipitated preparations of phosphorylated 15-kDa protein were dissolved in cleavage buffer containing 1:10 or 1:20 weight ratios of protease to substrate (estimated by 3P incorporation). Proteolysis by diphenylcarbamyl chloride-treated trypsin was conducted for 18 h at 37°C in 100 mM ammonium bicarbonate (pH 8.0), 10 mM CaCl\(_2\).

Digestion with *Staphylococcus aureus* V8 protease was performed in 50 mM ammonium acetate (pH 4.0) for 18 h at 37°C. Reactions were terminated with 0.1 M acetic acid, applied to 1-ml iron-affinity columns, and phosphorylated phosphopeptides eluted by increasing pH or phosphate gradients (19). Phosphopeptides eluting at pH 8.3-10.3 were further purified by high pressure liquid chromatography using a C\(_{18}\) reverse phase column equilibrated with 0.1% trifluoroacetic acid in water and developed with a linear gradient of 0.1% trifluoroacetic acid in acetonitrile.

**Amino Acid Sequence Analysis**—Protein and phosphopeptide sequences were determined using an Applied Biosystems model 470A gas-phase protein sequenator (20).

**Library Screening and cDNA Sequencing**—A canine cardiac AGT10 library (21) was plated in C600 HF Escherichia coli. Replicate nitrocellulose lifts were prehydrized for 3 h at 42°C in 6 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1 × Denhardt’s solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.1% SDS, and 100 μg denatured salmon sperm DNA/ml (22). The library was screened with two oligonucleotide probes based on reverse-translated amino-terminal (residues 4-14) and phosphopeptide (residues 54-61) sequences of the 15-kDa protein, consisting of all possible codon representations in positions of degeneracy. Oligonucleotides for screening were 5'-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP (22). Lifts were hybridized for 18 h at 42°C, then washed three times in 6 × SSC at 42°C for 30 min and in 2 × SSC at 42°C for 30 min. Cross-reacting positive clones identified by autoradiography were plaque-purified by three additional rounds of screening. Several putative 15-kDa clones were subcloned into the EcoRI site of pBluescript II SK vector (Stratagene) for nucleic acid sequence analysis. DNA sequencing was performed in both directions by the dideoxy method (23) using synthetic oligonucleotide primers and T7 DNA polymerase.

**Northern Blot Analysis**—Total RNA was isolated from different dog tissues (24) and transferred to nitrocellulose after electrophoresis of 10-μg aliquots in denaturing formaldehyde, 1.5% agarose gels (25). Northern blots were prehydrized for 3 h at 65°C in 50% formamide containing 4 × SSC, 5 × Denhardt’s solution, 250 μg denatured salmon sperm DNA/ml, 0.05 M sodium phosphate (pH 6.5), 0.1% SDS, 1 mM EDTA, and 1 mg/ml RNA. Full-length, anti-sense 32P-labeled RNA probe was synthesized by transcription using the linearized pBluescript II SK subclone as template, [α-32P]ATP, and T3 RNA polymerase (26). Blots were hybridized for 18 h at 65°C and then washed in 0.2 × SSC at 65°C for 30 min, 0.2% SDS at 65°C for 30 min, and twice in 0.1 × SSC, 0.1% SDS at 70°C for 30 min.

**Sequence Analyses**—Computer analyses of nucleic acid and protein sequences were performed using the PC Gene and IntelliGenetics computer programs. Hydropathy analysis was performed according to the procedure of Kyte and Doolittle (27) using a 19 amino acid window.

**RESULTS**

**15-kDa Protein Isolation**—The 15-kDa protein is the major substrate phosphorylated in sarcolemmal vesicles by endogenous PK-C (5) (Fig. 1, SL). Phosphorylated 15-kDa protein was purified from sarcolemmal vesicles by serial electrophoresis and electroelution, providing essentially complete recovery of radioactive protein at each step. The initial electroeluted sample contained a broad band of Coomassie Blue staining material with a mobility corresponding to that of the 15-kDa protein (Fig. 1, E1). A substantial purification was achieved by treating sample E1 with CNBr, which did not cleave the 15-kDa protein, but shifted other proteins in this region of the gel downward as cleavage fragments of increased mobility (Fig. 1, CBr). [32P]-Labeled protein was highly enriched in the final electroeluted sample and coincided with a single Coomassie Blue staining band (Fig. 1, E2).

**Amino Acid Sequence Determination**—Sample E2 was further purified by reverse-phase chromatography and subjected to automated Edman degradation. The single radioactive peak incorporated 32P, yielded a single sequence of 50 amino acid residues (Fig. 2A, line a). In other experiments, four proteolytic phosphopeptides (100-200 pmol each) were isolated and sequenced. Two of the tryptic phosphopeptides were limited peptides (Fig. 2A, lines b and c), whereas one was the product of incomplete digestion (Fig. 2A, line d). A single phosphopeptide was isolated from the V8 digest, whose sequence overlapped all three tryptic phosphopeptides (Fig. 2A, line e). More than 90% of the incorporated 32P was localized to the region encompassed by these phosphopeptides.

**Nucleic Acid Sequence Determination**—A canine left ventricular cDNA library was screened with two redundant oligonucleotide probes encoding for amino acid residues 4-14 and 54-61. Three cross-reacting positive clones were identified from 104 recombinant plaques. All three clones were similar in size and subsequently shown to code for the same protein. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 2B. The cDNA was 656 nucleotides long, consisting of a single open reading frame of 279 base pairs flanked by 102 and 275 base pairs of 5'- and 3'-untranslated sequence, respectively. An A at position −3 followed by a G at position +4, typical for translation initiation (28), surrounded the initiating ATG codon, which was in-frame with a downstream terminating TAG codon. A polyadenylation signal (29) near the 3' end was followed by a poly(A) tail of 70 nucleotides, suggesting that a full-length copy of the mRNA was sequenced.
PK-C are found (31). The phosphorylated forms of the protein mature protein is due. The protease phosphoptptide sequence. The signal sequence is numbered with +1 corresponding to the first base of the mature protein is shown in with negative values reflecting hydrophobicity. The lack of effect of CNBr on its mobility in SDS gels. This corresponds exactly to the protein sequence (Fig. 2A), demonstrating that we had sequenced the entire protein by standard Edman degradation techniques. cDNA sequencing also revealed that the amino acid sequence of the 15-kDa protein corresponded exactly to the protein sequence (Fig. 4).

**Protein Sequence Analysis**—The mature 15-kDa protein contained 72 amino acid residues with a calculated molecular weight of 8409. The deduced amino acid sequence (Fig. 2B) corresponded exactly to the protein sequence (Fig. 2A), demonstrating that we had sequenced the entire protein by standard Edman degradation techniques. cDNA sequencing also revealed that the protein contained a typical signal sequence (residues 1-20 through -1), with 2 basic residues located amino-terminally, followed by a hydrophobic core and a small aliphatic residue (alanine) at position -1 (30). The presence of a cleaved signal sequence is consistent with the amino terminus of the mature protein being unblocked. Likewise, the absence of methionine in the mature protein explained the lack of effect of CNBr on its mobility in SDS gels.

A distinguishing feature of the 15-kDa protein is its highly basic nature, with a calculated isoelectric point of 9.7. Most of the basic residues are concentrated at the carboxyl-terminal region, where consensus phosphorylation sites for PK-A and PK-C are found (31). The phosphorylated forms of the protein have neutral or slightly acidic isoelectric points (6).2

**Hydropathy analysis** (Fig. 2C) revealed the presence of a single hydrophobic domain of 20 uncharged amino acids (residues 18-37), sufficient to cross the sarcolemmal membrane, which separated the acidic amino-terminal end of the protein from the basic carboxyl-terminal end. A stop-transfer sequence, Arg-Arg-Cys-Arg-Cys-Lys (residues 38-43), was immediately adjacent to the transmembrane segment on the carboxyl side, consistent with the protein positioned in the membrane with its 35 carboxyl-terminal residues facing the cytoplasm (30). The 15-kDa protein can thus be defined as a class I, bitopic integral membrane protein by nature of its cleavable signal peptide and the stop-transfer sequence (30). A cartoon of the membrane topology of the 15-kDa protein is presented in Fig. 3.

**Nucleic Acid and Protein Sequence Data Bank Comparisons**—Comparison of the amino acid sequence of the cardiac 15-kDa protein with proteins in the National Biomedical Research Foundation Protein Identification Resources database revealed sequence similarity with the γ subunit, or “proteolipid,” of Na,K-ATPase isolated from sheep kidney (32). Residues 4-36 of the 15-kDa protein exhibited 52% identity with the partial sequence of the γ subunit (Fig. 4). Search of a recent release of the GenBank nucleic acid data base revealed no nucleic acid sequences homologous with the 15-kDa clone.

**Tissue Distribution of Cardiac 15-kDa mRNA—High-stringency Northern blot analysis was performed with the full-length antisense mRNA. A major hybridizing RNA species of about 700 nucleotides, approximating the size of the cDNA insert of the sequenced clone, was observed (Fig. 5, arrow).**

---

1 C. J. Palmer and L.R. Jones, unpublished observations.
The molecular weight of the 15-kDa protein determined from the deduced amino acid sequence (8,409) was substantially less than that estimated by SDS-PAGE. It is unlikely that the protein is a sulfhydryl-linked dimer, since its mobility in SDS-gels is not changed by boiling in the presence or absence of sulfhydryl-reducing agents. The nomenclature of “15-kDa protein” (5, 6, 13) or “16-kDa protein” (8, 9, 11) used previously for this entity is inappropriate when, in fact, the actual molecular mass is approximately one-half this value. Therefore, we propose the name “phospholemman” for this protein, indicating a major phosphoprotein substrate localized to the plasmalemma.

The amino acid sequence of phospholemman was consistent with previous studies suggesting multiple, separate sites phosphorylated by at least two protein kinases. The carboxyl-terminal region of phospholemman contained predicted phosphorylation sites for PK-A and PK-C, as well as for cGMP-dependent and Ca²⁺/calmodulin-dependent protein kinases (31). Our own studies, however, revealed that the protein was not a substrate for the latter two kinases, which is consistent with the results of Walaas et al. (6, 7), who examined the phosphoprotein in skeletal muscle plasma membranes. We observed only serine phosphorylation of phospholemman using PK-A, whereas PK-C phosphorylated both serine and threonine residues (data not shown).

The mRNA transcript of phospholemman was not limited to heart but was present in all muscle types as well as in liver. An interesting finding was the lack of significant phospholemman mRNA in dog kidney and brain, even though phospholemman shares sequence similarity with the γ subunit of Na,K-ATPase isolated from sheep kidney. Brain and kidney contain relatively high levels of Na,K-ATPase and so would be predicted to contain high levels of γ subunit. The inability of the probe to hybridize with an mRNA species in brain and kidney could mean that the γ subunit and phospholemman are products of different genes, an idea supported by comparison of our cDNA sequence to the kidney γ subunit cDNA sequence. The γ subunit of Na,K-ATPase is not known to be phosphorylated, and it may be that phospholemman has evolved specifically to allow regulation by multisite phosphorylation (1–14).

Phospholemman shows many similarities to the regulatory protein, phospholamban, which is localized to sarcoplasmic reticulum in heart and modulates the activity of the sarcoplasmic reticulum calcium pump (33, 34). Both proteins are small, highly basic, and possess single membrane-spanning domains consisting entirely of uncharged residues (35). Both proteins are oriented with their most positively charged regions directed toward the cytoplasm, where the sites of phosphorylation are localized (35). Multisite phosphorylation of both proteins occurs in intact myocardium by protein kinases which are activated by cAMP and calcium (13, 14, 36), and phosphorylation of both proteins produces substantial changes in charge, with isoelectric points changing from approximately 10 to 5 or 6 (37). A short region of sequence similarity between phospholamban and phospholemman, where 7 out of 9 residues are identical, is especially intriguing.

| Phospholemman | R S S T R L S T R R³² |
| Phospholamban | R S A I R R A S T I E M³⁹ |

Ser³⁶ and Thr³⁷ of phospholamban in this region are phosphorylated exclusively by PK-A and multifunctional Ca²⁺/calmodulin-dependent protein kinase, respectively (35). Consensus phosphorylation site data (31) suggest that Ser³⁶ of phospholemman is a prime target for PK-A phosphorylation. Although phospholemman is not a substrate for Ca²⁺/calmodulin-dependent protein kinase, the position of 3 consecutive carboxyl-terminal arginine residues (residues 70–72), as well as arginine residues 61, 65, and 66, suggest that serine residues 62, 63, and 68 and threonine residue 69 are all potential substrates for PK-C. Several peptides encompassing residues 61–72 were isolated as phosphopeptides, demonstrating that serine and threonine residues in this region of the molecule are phosphorylated. As suggested for phospholamban (38), it is possible that alteration of membrane surface charge secondary to phospholemman phosphorylation may play a role in phospholemman function. Phosphorylation-induced perturbation of sarcolemmal surface charge could alter the local calcium concentration, with resultant effects on activities of co-localized channels, pumps, and/or antiporters. Recently, a cardiac delayed rectifier L₅ channel has been cloned and sequenced (39), which shows some structural similarity to phospholemman. Both proteins are small and traverse the sarcolemmal membrane once. The membrane-spanning regions of both proteins are enriched in glycine and contain no charged residues. The possibility that phospholemman is a channel should also be considered.

Acknowledgments—We thank Joyce Dwulet for protein and peptide sequencing, Lisa Lewis for secretarial assistance, and Steve Cala for providing helpful comments on the manuscript. We also thank B. Forbush and R. Mercer for access to the cDNA sequence of the γ subunit of Na,K-ATPase.

³ B. Forbush and R. Mercer, personal communication.
REFERENCES

1. Jones, L. R., Besch, H. R., Jr., Fleming, J. W., McConnaughey, M. M., and Watanabe, A. M. (1979) J. Biol. Chem. 254, 530-539
2. Jones, L. R., Maddock, S. W., and Besch, H. R., Jr. (1980) J. Biol. Chem. 255, 9971-9980
3. Jones, L. R., Maddock, S. W., and Hathaway, D. R. (1981) Biochim. Biophys. Acta 641, 242-253
4. Manalan, A. S., and Jones, L. R. (1982) J. Biol. Chem. 257, 5052-5057
5. Presti, C. F., Scott, B. T., and Jones, L. R. (1985) J. Biol. Chem. 260, 13879-13889
6. Walaas, S. I., Horn, R. S., Albert, K. A., Adler, A., and Walaas, O. (1988) Biochim. Biophys. Acta 968, 127-137
7. Walaas, S. I., Horn, R. S., Nairn, A. C., Walaas, O., and Adler, A. (1988) Arch. Biochem. Biophys. 262, 245-258
8. Boulanger-Saunier, C., Kattenberg, D. M., and Stoclet, J.-C. (1987) Biochem. Biophys. Res. Commun. 143, 517-524
9. Sarcevic, B., Brookes, V., Martin, T. J., Kemp, B. E., and Robinson, P. J. (1989) J. Biol. Chem. 264, 20648-20654
10. Cooper, R. H., Kobayashi, K., and Williamson, J. R. (1984) FEBS Lett. 166, 125-130
11. Widmaier, E. P., Osawa, S., and Hall, P. F. (1986) Endocrinology 118, 701-708
12. Presti, C. F., Jones, L. R., and Lindemann, J. P. (1985) J. Biol. Chem. 260, 3860-3867
13. Lindemann, J. P. (1986) J. Biol. Chem. 261, 4860-4867
14. Jones, L. R. (1988) Methods Enzymol. 157, 85-91
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
16. Laemmli, U. K. (1970) Nature 227, 680-685
17. Wessel, D., and Flugge, U. I. (1984) Anal. Biochem. 138, 141-143
18. Anderson, L., and Porath, J. (1986) Anal. Biochem. 154, 250-254
19. Cala, S. E., and Jones, L. R. (1991) J. Biol. Chem. 266, 391-398
20. Scott, B. T., Simmerman, H. K., Collins, J. H., Nadal-Ginard, B., and Jones, L. R. (1988) J. Biol. Chem. 263, 8965-8964
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
23. Solero, R., Birnboim, H., and Darnell, J. (1966) J. Mol. Biol. 19, 362-373
24. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201-5205
25. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) Nucleic Acids Res. 12, 7035-7056
26. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132
27. Walaas, S. I., and Brownlee, G. G. (1976) Nature 265, 211-214
28. Von Heijne, G. (1990) J. Membr. Biol. 115, 195-201
29. Kemp, B., and Pearson, R. B. (1990) Trends Biochem. Sci. 15, 342-346
30. Collins, J., and Leszczynski, J. (1987) Biochemistry 26, 8665-8668
31. Tada, M., and Kadoma, M. (1989) BioEssays 10, 157-163
32. Jorgensen, A. O., and Jones, L. R. (1986) J. Cell Biol. 261, 3775-3781
33. Wegener, A. D., Lindemann, J. P., Simmerman, H. K., and Jones, L. R. (1989) J. Biol. Chem. 264, 11468-11474
34. Jones, L. R., Simmerman, H. K., Bos, S. V., Wilson, W. W., and Wegener, A. D. (1985) J. Biol. Chem. 260, 7721-7730
35. Folander, K., Smith, J. S., Antanavage, J., Bennett, C., Stein, R. B., and Swanson, R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2975-2979