Torrie, G. M., & Valleau, J. P. (1977) *J. Comput. Phys. 23*, 187-199.
Warshel, A. (1978) *Chem. Phys. Lett. 55*, 454-458.
Warshel, A. (1982) *J. Phys. Chem. 86*, 2218-2224.
Warshel, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 444-448.
Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227-249.
Warshel, A., & Russell, S. T. (1984) *Q. Rev. Biophys. 17*, 283-422.
Warshel, A., & King, G. (1985) *Chem. Phys. Lett. 121*, 124-129.
Warshel, A., & Sussman, F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3806-3810.

Domain Organization of Chicken Gizzard Myosin Light Chain Kinase Deduced from a Cloned cDNA†

Vince Guerriero, Jr.,‡ Mario A. Russo,§ Norma J. Olson, John A. Putkey,∥ and Anthony R. Means*
Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030
Received April 23, 1986; Revised Manuscript Received August 14, 1986

ABSTRACT: Myosin light chain kinases (MLCK) are the most studied of the calmodulin-activated enzymes; however, minimal sequence information is available for the smooth muscle form of the enzyme. The production of an antibody against the enzyme and the use of expression vectors for constructing cDNA libraries have facilitated the isolation of a cDNA for this kinase. The derived amino sequence was found to contain a region of high homology (54%) to the rabbit skeletal muscle enzyme and also very significant homology (35%) to the catalytic subunit of phosphorylase *b* kinase and cGMP-dependent protein kinase. All of these homologies were found in the known catalytic domains of these enzyme, thus enabling us to predict the location of the catalytic domain for the chicken gizzard myosin light chain kinase. Within the catalytic domain a consensus sequence for an ATP-binding site was located. Subcloning and expression of different regions of the cDNA defined a 192 base pair fragment coding for the calmodulin-binding domain of MLCK. Both of the cAMP-dependent protein kinase phosphorylation sites were identified by sequence homology. A linear model for MLCK is presented placing the various domains in relative position. Northern blot analysis and S_1 protection and mapping experiments have revealed that the mRNA for MLCK is 5.5 kilobases in length, but there also exists a second mRNA of 2.7 kilobases that shares a high degree of homology with about 520 base pairs at the 3′ end of the cDNA for MLCK.

Myosin light chain kinases (MLCK) are Ca^{2+}-calmodulin-dependent enzymes that phosphorylate the regulatory light chain of myosin. In smooth muscle and nonmuscle tissues this phosphorylation is obligatory for the stimulation of myosin ATPase, which precedes tension development (Adelstein & Klee, 1980). Myosin light chain kinases have been isolated from a variety of sources including smooth muscle, skeletal muscle, cardiac muscle, platelets, and macrophages. The enzymes are substrate-specific and preferentially phophorylate light chains from homologous tissues. One common characteristic these enzymes share is the requirement of calmodulin for activity.

These enzymes are also phosphorylated by cAMP-dependent protein kinase. The smooth muscle (Conti & Adelstein, 1981) and nonmuscle forms (Nishikawa et al., 1984) of the enzyme are inhibited by phosphorylation due to a decreased affinity for calmodulin. The cardiac (Wolf & Hofman, 1980) and skeletal muscle (Edelman & Krebs, 1982) forms are also phosphorylated, but this has no effect on either enzyme activity or calmodulin affinity. Finally, the enzyme from the striated muscle of *Limulus* (Sellers & Harvey, 1984) is not phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

These differences in various myosin light chain kinase enzymes must arise from alterations in the primary structure of the proteins. To date, the only MLCK to be extensively sequenced is from rabbit skeletal muscle (Takio et al., 1985). The organization of the functional domains in this enzyme are similar to those predicted for the chicken smooth muscle MLCK from the proteolysis mapping studies of Foyt et al. (1985). As a further step in understanding the relationship between the primary structure and location of functional domains of these enzymes, we have cloned and sequenced a cDNA encoding the carboxy-terminal 60% of chicken gizzard MLCK. Comparison of this sequence with that of the rabbit skeletal enzyme reveals a high degree of homology in the predicted catalytic site. Indeed the similarity of this region with identical sized portions of other protein kinases suggests a common evolutionary history.

Materials and Methods

Antibody to MLCK was produced and purified as described by Guerriero et al. (1981). Protein A was iodinated by the Bolton-Hunter procedure (1973) as modified by Chafouleas et al. (1979).

Preparation of Poly(A+) RNA. The muscular portion of chicken gizzards was cut into small pieces (approximately 3-5

† This work was supported by National Institutes of Health Grant HD-07503 (A.R.M.), American Cancer Society Grant BC-326 (A.R.M.), Robert A. Welch Foundation Grant Q-611 (A.R.M.), and USPHS Grant RR-05425 (V.G.).
* Address correspondence to this author.
‡ Present address: Department of Animal Sciences, University of Arizona, Tucson, AZ 85721.
§ Present address: Instituto di Isologia ed Embriologia Generale, Universita di Roma, Rome, Italy.
∥ Present address: Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, TX 77225.

0006-2960/86/0425-8372$01.50/0 © 1986 American Chemical Society

mm) and stored frozen in liquid nitrogen. The frozen tissue was pulverized by pounding between two lead bricks lined with aluminum foil, and total RNA was isolated as described by Rosen et al. (1975). Poly(A+) RNA was then isolated according to the procedure of Aviv and Leder (1972).

Construction of cDNA Library and Screening with Antibody. Double-stranded cDNA was prepared according to the procedure of Gubler and Hoffman (1983). The cDNA was methylated with the procedure of Maniatis et al. (1978). The reaction volume was 75 μL and contained 0.1 M Tris-HCl[1] (pH 8.0), 10 mM EDTA, 6 μM *S*-adenosyl-L-methionine, and 60 units of *Eco*RI methylase (New England Bio Labs). Following incubation at 37 °C for 60 min, the products were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol-precipitated from 2 M ammonium acetate. *Eco*RI linkers were then added, and the double-stranded DNA was digested with *Eco*RI to create cohesive ends. The DNA was then size fractionated on a 1% low-melting agarose gel, and the 1–3-kb fraction was ligated into the expression phage λgt11 described by Young and Davis (1983a). The recombinant DNAs were packaged into phage by using the packaging extracts from Amersham. The library contained 50 000 members with 85% containing inserts.

The library was screened with affinity-purified polyclonal antibodies to chicken gizzard MLCK (Guerriero et al., 1981) as described by Young and Davis (1983b). Plaques corresponding to positive signals were picked and rescreened until signals were homogenous.

Purification of Antibodies Using Expressed Antigens. *Escherichia coli* strain Y1090 was infected with the specified purified phage and plated in top agar. Plates were incubated at 42 °C for 2–4 h and then overlayed with a nitrocellulose filter that had been saturated with 10 mM IPTG. The plate was incubated for 4–5 h at 37 °C during which time the cloned cDNA was expressed and the protein was deposited on the filter. The filters were then incubated with a 3% bovine serum albumin solution in Tris–saline. After a brief rinse with Tris–saline, the filters were incubated with antibody against MLCK (20 μg/mL) overnight. The filters were then rinsed with Tris–saline for 2 h with three to five changes of buffer. The bound antibody was eluted from the filter by incubation with 5 mL of elution buffer (100 mM glycine hydrochloride, pH 2.7, and 75 mM NaCl) for 2 min. The antibody was then dialyzed overnight against borate-buffered saline (100 mM boric acid, 25 mM sodium borate, and 75 mM NaCl).

Sequencing. Restriction fragments of pMK 2.1 (MK 2.1 was first subcloned in pUC8) were subcloned into M13mp18 or M13mp19 (Messing, 1983). The nucleotide sequence was determined by the dideoxynucleotide chain termination method of Sanger et al. (1977). The 3′ and 5′ end sequences were confirmed by the Maxim–Gilbert method (Maxim & Gilbert, 1977).

Sequence Analysis and Homology Search. The program described by Queen and Korn (1984) and supplied by Beckman (MicroGenie) was used for sequence analysis. The national Biomedical Research Foundation (NBRF) Protein Data Bank was searched for homologies.

Production and Analysis of Fusion Proteins. The procedure used was derived from that Young and Davis (1983b). *E. coli* strain Y1090 (500 000 cells) was infected with plaque-purified phage at a multiplicity of infection of 1 for 30 min at 32 °C. The cells were incubated in 25 mL of media at 32 °C with shaking and grown to an A_{550nm} of approximately 0.2. The bacteria were incubated at 42 °C for 15 min and aerated by shaking at 37 °C for 2 h in the presence of 2 mM IPTG. The cells were then pelleted by centrifugation, suspended in 0.5 mL of sample buffer (Laemmli, 1970), and passed through a 21-gauge needle several times. The samples were then heated at 90 °C for 5 min and centrifuged to remove insoluble material. Aliquots were analyzed on 7.5% polyacrylamide slab gels containing sodium dodecyl sulfate (Laemmli, 1970). Protein staining of gels was done with Coomassie Blue G as described by Foyt et al. (1985). Samples used for Western analysis were transferred onto nitrocellulose paper and analyzed with anti-MLCK antibody as previously described by Guerriero et al. (1981). Calmodulin-binding activity was assessed as described by Foyt et al. (1985).

Construction of Subclones. The expression vector pUC19 (P-L Biochemicals) was modified to prevent the cloned expressed protein from fusing to β-galactosidase by insertion of a universal terminator sequence (Pharmacia) into the *Sma*I site in the multiple cloning site. This modified vector is referred to as pUC19T. In all cases, the cDNA was inserted consistent with the open reading frame shown in Figure 2. In either of the other two reading frames, no protein product was produced.

Construction of pMK 470 (Figure 5) was accomplished by first cutting pMK 2.1 with *Sst*I and then blunt ending with T4 DNA polymerase. The plasmid was then digested with *Eco*RI and blunt-ended with the Klenow fragment of DNA polymerase I. The blunt *Sst*I was ligated to the blunt-ended (Klenow-treated) *Hin*dIII site of pUC19T. The blunt ended *Eco*RI site was ligated to the blunt-ended *Sal*I site (Klenow treated) of pUC19T.

The construct pMK 540 (Figure 5) consisted of a fragment from MK 2.1 starting at the *Hin*cII site near the center of the cDNA and extending to the *Sst*I site (starting at nucleotide 1578) at the 3′ end. This *Sst*I site was blunt-ended with T4 DNA polymerase and ligated to the blunt-end *Hin*cII site of pUC19T. The *Pst*I site of pUC19T was blunt-ended with T4 DNA polymerase and ligated to the *Hin*cII site at the 5′ end of the insert.

To construct pMK 770 (Figure 5), the *Rsa*I site (nucleotide 1385) of MK 2.1 was ligated to the blunt-ended *Pst*I site in pUC19T. The *Eco*RI site at the 3′ end of the insert was filled in with the Klenow fragment of DNA polymerase I and ligated to the *Sal*I site of pUC19T that also had been filled in with Klenow fragment of DNA polymerase I to create a blunt end.

Expression and Analysis of Subcloned Fragments. Single colonies were picked and grown overnight in 5 mL of culture media containing 2 mM IPTG. The cells were then pelleted and lysed in 0.5 mL of gel sample buffer (Laemmli, 1970), passed through a 21-gauge needle several times, heated at 90 °C for 10 min, and centrifuged to remove insoluble material.

Aliquots were analyzed on 12–20% exponential-gradient polyacryamide gels (Laemmli, 1970). Proteins were then electrophoretically transferred to nitrocellulose paper. For immunological detection of proteins, the nitrocellulose sheets were treated as described by Guerriero et al. (1981) except that 5% nonfat dry milk was substituted for bovine serum albumin in all buffers. Samples that were assayed for calmodulin binding were first rinsed for 10 min in 0.1 M imidazole, pH 7.0. Unoccupied protein binding sites on the paper were saturated by incubation in solution G (20 mM imidazole, pH 7.0, 0.2 M KCl, 5% nonfat dry milk, and 1 mM Ca^{2+} or 2 mM EGTA) for 1 h. The sheets were then incubated in

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thio-β-D-galactopyranoside; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; kb, kilobase(s); bp, base pair(s).

solution G containing 1×10^6 cpm of ^{125}I-labeled calmodulin/mL for 2 h and then rinsed with solution G (without the milk) for a total of 1 h with five to ten buffer changes. The nitrocellulose sheets were air-dried and exposed to X-ray film.

Transformations. All plasmid constructs were transformed into *E. coli* K-12 strain JM 103 by the procedure of Hanahan (1983).

Northern Blot Analysis. Poly(A+) RNA (10 μg/lane) was electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to Biodyne A membrane (1.2-μm pore size, Pall, Glen Cove, NY), and hybridized to ^{32}P-labeled cDNAs.

Preparation of cDNA Probes. The MLCK sequences were derived from MK 2.1, MK 0.8, or MK 0.35. The plasmids were cut with the appropriate restriction enzymes and electrophoresed in a low melting temperature agarose gel (Bio-Rad). The DNA band containing the desired fragment was excised from the gel and labeled by the random oligopriming method of Feinberg and Volgelstein (1983). The incorporation of ^{32}P (0.05 mCi, 3000 Ci/mmol) ranged from 40 to 80% in 30–70 ng of DNA depending on the fragment used. All labeled probes were separated from free [^{32}P]dCTP by Sephadex G-75 column chromatography.

S_1 Protection and Mapping. The entire insert from pMK 2.1 was excised by cutting with *Eco*RI and separated from the vector by electrophoresis through a low melting temperature agarose gel. The fragment was cut from the gel, and the agarose was removed with the phenol extraction method described by Maniatis et al. (1982). The insert (75 ng) was then added to a hybridization mixture (total volume of 100 μL) containing 70% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 8.8 μg of poly(A+) RNA from chicken gizzard or 10 μg of tRNA. The mixtures were heated to 80 °C for 10 min and then cooled to the indicated temperature for overnight incubation. The reactions were stopped by the addition of ice-cold S_1 buffer (final concentrations of 200 mM sodium acetate, pH 4.5, 500 mM NaCl, 2 mM Zn_2SO_4, and 500 units of S_1 nuclease in a volume of 500 μL). Following incubation at 37 °C for 90 min, the samples were precipitated by the addition of 20 μg of glycogen (Boehringer-Mannheim) as carrier and 2 volumes of 95% ethanol. Pellets were rinsed with 70% ethanol and then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide before Southern blotting. Two additional protection experiments were carried out on fragments derived from pMK 2.1. The first was a 360-nucleotide *Eco*RI/*Bgl*II piece from the 5′ end of the clone. The second was an internal *Taq*I fragment including nucleotides 1296–1740. For S_1 mapping, pMK 2.1 was digested with either *Bgl*II or *Taq*I, treated with bacterial alkaline phosphatase, and 5′ end-labeled with T_4 polynucleotide kinase. The DNA samples were then digested with either *Hin*fI or *Eco*RI, and the appropriate fragments were isolated by gel electrophoresis. The specific activity was in each case approximately 1600 cpm/ng, and 80 000 cpm was used per hybridization with the conditions described above for S_1 protection. Samples were analyzed on a 5% polyacrylamide gel containing 7 M urea. Molecular weight standards consisted of end-labeled fragments of pBR 322 cut with *Hpa*II. The 263- and 254-bp fragments also used as standards in Figure 8B were derived from various restriction sites within pMK 2.1.

Southern Blotting. The DNA/RNA hybrids from the S_1 protection experiments were denatured by soaking the gel in several volumes of 0.5 M NaOH and 1.5 M NaCl for 1 h. The gel was neutralized for 1 h in 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl. The samples were transferred to Biodyne A overnight with the Southern transfer procedure (Southern, 1975). The blot was probed with MK 2.1 as described for Northern analyses. Additional Southern analyses were carried out by removing the 800-bp insert from pMK 0.8, electrophoresing on a 1% agarose gel, and probing with the oligolabeled insert from pMK 0.35. In addition, pMK 2.1 was digested with *Hin*cII, and the fragments were separated on a 1% agarose gel and probed with the oligolabeled insert from pMK 0.80. Transfer to Biodyne A and hybridization conditions were as described above.

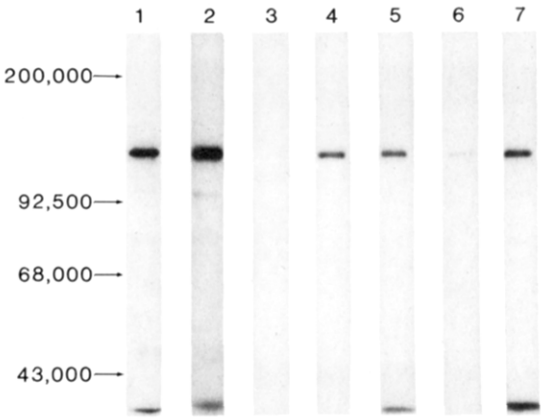
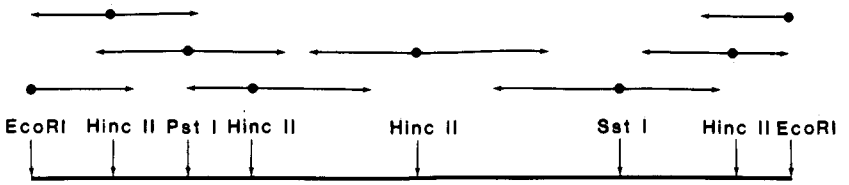


FIGURE 1: Western blots using clone affinity purified antibodies. Purified MLCK (lane 1, 20 ng) or total gizzard homogenates (lanes 2–7) were electrophoresed on a 7.5% polyacramide gel and electrophoretically transferred to nitrocellulose. The blots were probed with total anti-MLCK antibody (lanes 1 and 2) or with antibody affinity purified with the following clones: λgt11 with no insert (lane 3), MK 0.12 (lane 4), MK 1.0 (lane 5), MK 0.35 (lane 6), and MK 2.1 (lane 7). The positions and sizes of molecular weight markers are indicated on the left. The additional signal at the bottom of some lanes is in the dye front. Whereas these signals may represent interaction of the antibody with small degradation products of MLCK, we cannot identify them with certainty.

RESULTS

Screening of the chicken gizzard library resulted in several positive clones identified as MK 2.1, MK 1.0, MK 0.35, and MK 0.10 (numbers indicate the size of the insert in kilobases). As an initial test to determine if these clones were for MLCK, the protein expressed in bacteria was used to affinity purify antibody against MLCK. This clone affinity purified antibody was then used to probe a Western blot of total chicken gizzard proteins. The results (Figure 1) demonstrate that in each case the clone affinity purified antibody bound to a protein of approximately 130 000 daltons in size (Figure 1, lanes 4–7), which comigrated with purified MLCK (Figure 1, lane 1). Lane 3 is a control in which the antibody was purified from bacteria lysates infected with vector without an insert.

The longest clone, MK 2.1, was subcloned into the sequencing vector M13 and sequenced by the strategy outlined in Figure 2. The complete nucleotide sequence of MK 2.1 and the corresponding translated amino acid sequence are presented in Figure 2. There is an open reading frame coding for 655 amino acids (nucleotides 43–2007). MLCK is a protein of approximately 130 000 daltons in size, so it would contain about 1130 amino acids. The termination codon at the 3′ end of the cDNA has been verified by sequencing an overlapping clone (MK 1.0). There is, however, another termination codon at the 5′ end beginning at nucleotide 40. The sequence in this region has been confirmed by the method of Maxam and Gilbert (1977). We attempted to determine if this apparent stop codon was an artifact by carrying out an mRNA protection experiment using an end-labeled 360-bp *Eco*RI to *Bgl*II fragment of pMK 2.1. Following hybridization




```
                                30                                  60
AAT TCC GCT CTT TTT GAA GCC ACT TGT TCT ACC AAA TGT TAG TGC ACC ATA ATT TCC ACC
                                                   END
                                10                                  20

                                90                                 120
TGG ATG AAA TTC CGT AAG CAG ATT CAA GAA AAT GAA TAT ATC AAA ATT GAG AAT GCT GAA
Trp Met Lys Phe Arg Lys Gln Ile Gln Glu Asn Glu Tyr Ile Lys Ile Glu Asn Ala Glu
                                30                                  40

                               150                                 180
AAC AGC AGC AAG CTA ACA ATA TCA TCG ACA AAG CAG GAA CAC TGC GGA TGT TAC ACG CTA
Asn Ser Ser Lys Leu Thr Ile Ser Ser Thr Lys Gln Glu His Cys Gly Cys Tyr Thr Leu
                                50                                  60

                               210                                 240
GTT GTA GAA AAC AAA CTT GGC AGC AGA CAA GCA CAA GTC AAC CTT ACA GTT GTT GAT AAA
Val Val Glu Asn Lys Leu Gly Ser Arg Gln Ala Gln Val Asn Leu Thr Val Val Asp Lys
                                70                                  80

                               270                                 300
CCA GAC CCA CCT GCT GGA ACG CCT TGT GCA TCA GAC ATA CGG AGT TCT TCC CTC ACT CTT
Pro Asp Pro Pro Ala Gly Thr Pro Cys Ala Ser Asp Ile Arg Ser Ser Ser Leu Thr Leu
                                90                                 100

                               330                                 360
TCA TGG TAC GGC TCC TCT TAT GAT GGT GGA AGT GCG GTG CAG TCC TAC ACT GTG GAG ATC
Ser Trp Tyr Gly Ser Ser Tyr Asp Gly Gly Ser Ala Val Gln Ser Tyr Thr Val Glu Ile
                               110                                 120

                               390                                 420
TGG AAC TCA GTG GAT AAC AAA TGG ACA GAC CTC ACT ACC TGC CGC AGC ACG TCT TTC AAC
Trp Asn Ser Val Asp Asn Lys Trp Thr Asp Leu Thr Thr Cys Arg Ser Thr Ser Phe Asn
                               130                                 140

                               450                                 480
GTG CAG GAC CTG CAG GCT GAC CGG GAG TAC AAA TTC CGT GTA CGA GCT GCT AAC GTG TAC
Val Gln Asp Leu Gln Ala Asp Arg Glu Tyr Lys Phe Arg Val Arg Ala Ala Asn Val Tyr
                               150                                 160

                               510                                 540
GGC ATC AGT GAG CCC AGT CAA GAA TCT GAG GTG GTA AAA GTT GGA GAG AAA CAA GAA GAA
Gly Ile Ser Glu Pro Ser Gln Glu Ser Glu Val Val Lys Val Gly Glu Lys Gln Glu Glu
                               170                                 180

                               570                                 600
GAA CTT AAA GAG GAG GAA GCA GAG CTG TCT GAC GAT GAA GGC AAA GAA ACA GAG GTC AAC
Glu Leu Lys Glu Glu Glu Ala Glu Leu Ser Asp Asp Glu Gly Lys Glu Thr Glu Val Asn
                               190                                 200

                               630                                 660
TAT CAG ACA GTT ACT ATC AAC ACC GAA CAG AAA GTT TCA GAT GTC TAT AAT ATT GAA GAA
Tyr Gln Thr Val Thr Ile Asn Thr Glu Gln Lys Val Ser Asp Val Tyr Asn Ile Glu Glu
                               210                                 220

                               690                                 720
AGA CTG GGA TCG GGG AAA TTT GGA CAA GTC TTT AGG CTT GTT GAA AAG AAG ACT GGA AAA
Arg Leu Gly Ser Gly Lys Phe Gly Gln Val Phe Arg Leu Val Glu Lys Lys Thr Gly Lys
                               230                                 240

                               750                                 780
GTG TGG GCA GGA AAA TTT TTC AAA GCA TAT TCT GCA AAG GAA AAA GAG AAC ATA AGG GAT
Val Trp Ala Gly Lys Phe Phe Lys Ala Tyr Ser Ala Lys Glu Lys Glu Asn Ile Arg Asp
                               250                                 260

                               810                                 840
GAA ATC AGC ATC ATG AAC TGT CTG CAT CAC CCA AAG CTC GTG CAA TGT GTA GAT GCC TTT
Glu Ile Ser Ile Met Asn Cys Leu His His Pro Lys Leu Val Gln Cys Val Asp Ala Phe
                               270                                 280

                               870                                 900
GAA GAA AAA GCC AAC ATT GTT ATG GTC TTG GAA ATG GTT TCT GGA GGA GAA CTC TTT GAA
Glu Glu Lys Ala Asn Ile Val Met Val Leu Glu Met Val Ser Gly Gly Glu Leu Phe Glu
                               290                                 300

                               930                                 960
CGT ATC ATT GAT GAA GAC TTT GAA CTG ACA GAG AGA GAA TGC ATT AAA TAT ATG AGG CAG
Arg Ile Ile Asp Glu Asp Phe Glu Leu Thr Glu Arg Glu Cys Ile Lys Tyr Met Arg Gln
                               310                                 320

                               990                                1020
ATT TCA GAA GGA GTT GAG TAC ATC CAT AAG CAG GGT ATT GTC CAT TTG GAT TTG AAG CCA
Ile Ser Glu Gly Val Glu Tyr Ile His Lys Gln Gly Ile Val His Leu Asp Leu Lys Pro
                               330                                 340
```

```
                              1050                                1080
GAA AAT ATT ATG TGT GTC AAC AAG ACT GGT ACA AGT ATC AAA CTC ATT GAC TTC GGA CTT
Glu Asn Ile Met Cys Val Asn Lys Thr Gly Thr Ser Ile Lys Leu Ile Asp Phe Gly Leu
                               350                                 360

                              1110                                1140
GCA AGA AGA TTA GAA AGT GCA GGT TCT CTC AAA GTT CTC TTT GGG ACA CCT GAG TTT GTG
Ala Arg Arg Leu Glu Ser Ala Gly Ser Leu Lys Val Leu Phe Gly Thr Pro Glu Phe Val
                               370                                 380

                              1170                                1200
GCT CCA GAA GTT ATA AAC TAT GAA CCT ATC GGA TAT GAA ACA GAT ATG TGG AGT ATA GGA
Ala Pro Glu Val Ile Asn Tyr Glu Pro Ile Gly Tyr Glu Thr Asp Met Trp Ser Ile Gly
                               390                                 400

                              1230                                1260
GTT ATC TGC TAC ATC CTG GTC AGC GGG CTT TCT CCC TTC ATG GGA GAC AAC GAC AAC GAG
Val Ile Cys Tyr Ile Leu Val Ser Gly Leu Ser Pro Phe Met Gly Asp Asn Asp Asn Glu
                               410                                 420

                              1290                                1320
ACC CTG GCC AAC GTC ACC TCT GCC ACG TGG GAC TTC GAT GAT GAG GCT TTT GAT GAA ATC
Thr Leu Ala Asn Val Thr Ser Ala Thr Trp Asp Phe Asp Asp Glu Ala Phe Asp Glu Ile
                               430                                 440

                              1350                                1380
TCT GAC GAT GCC AAG GAC TTT ATC AGC AAT CTC CTG AAG AAA GAC ATG AAG AGT CGC TTA
Ser Asp Asp Ala Lys Asp Phe Ile Ser Asn Leu Leu Lys Lys Asp Met Lys Ser Arg Leu
                               450                                 460

                              1410                                1440
AAT TGT ACT CAA TGT CTT CAG CAC CCG TGG CTG CAA AAA GAC ACC AAA AAC ATG GAA GCC
Asn Cys Thr Gln Cys Leu Gln His Pro Trp Leu Gln Lys Asp Thr Lys Asn Met Glu Ala
                               470                                 480

                              1470                                1500
AAA AAA CTT TCC AAA GAT AGG ATG AAG AAA TAT ATG GCC AGA AGA AAA TGG CAG AAA ACA
Lys Lys Leu Ser Lys Asp Arg Met Lys Lys Tyr Met Ala Arg Arg Lys Trp Gln Lys Thr
                               490                                 500

                              1530                                1560
GGC CAT GCT GTC CGA GCA ATA GGA AGA CTG TCA TCC ATG GCA ATG ATT TCT GGT ATG AGT
Gly His Ala Val Arg Ala Ile Gly Arg Leu Ser Ser Met Ala Met Ile Ser Gly Met Ser
                               510                                 520

                              1590                                1620
GGG AGG AAG GCC TCT GGG AGC TCG CCT ACC AGC CCG ATA AAT GCA GAC AAA GTA GAG AAC
Gly Arg Lys Ala Ser Gly Ser Ser Pro Thr Ser Pro Ile Asn Ala Asp Lys Val Glu Asn
                               530                                 540

                              1650                                1680
GAA GAT GCC TTC CTT GAA GAA GTG GCT GAG GAA AAG CCC CAT GTA AAG CCA TAC TTT ACT
Glu Asp Ala Phe Leu Glu Glu Val Ala Glu Glu Lys Pro His Val Lys Pro Tyr Phe Thr
                               550                                 560

                              1710                                1740
AAA ACA ATC CTT GAC ATG GAG GTT GTG GAA GGA AGT GCT GCT AGG TTT GAC TGC AAA ATC
Lys Thr Ile Leu Asp Met Glu Val Val Glu Gly Ser Ala Ala Arg Phe Asp Cys Lys Ile
                               570                                 580

                              1770                                1800
GAA GGC TAT CCT GAC CCT GAG GTA ATG TGG TAC AAA GAT GAT CAG CCT GTC AAG GAG TCC
Glu Gly Tyr Pro Asp Pro Glu Val Met Trp Tyr Lys Asp Asp Gln Pro Val Lys Glu Ser
                               590                                 600

                              1830                                1860
CGT CAC TTC CAG ATA GAT TAT GAT GAG GAA GGG AAC TGT TCT TTG ACT ATT TCT GAA GTA
Arg His Phe Gln Ile Asp Tyr Asp Glu Glu Gly Asn Cys Ser Leu Thr Ile Ser Glu Val
                               610                                 620

                              1890                                1920
TGT GGG GAC GAT GAT GCC AAA TAC ACC TGC AAA GCT GTT AAC AGC CTT GGG GAA GCC ACA
Cys Gly Asp Asp Asp Ala Lys Tyr Thr Cys Lys Ala Val Asn Ser Leu Gly Glu Ala Thr
                               630                                 640

                              1950                                1980
TGT ACC GCA GAA CTC CTT GTG GAA ACA ATG GGA AAA GAG GGA GAA GGA GAA GGA GAA GGA
Cys Thr Ala Glu Leu Leu Val Glu Thr Met Gly Lys Glu Gly Glu Gly Glu Gly Glu Gly
                               650                                 660

                                                2010                2040
GAA GAA GAT GAA GAG GAG GAG GAA GAA TGAAACAAGGCTCAAAGAAAACAAAAAAAAAAAAAGGAATTC
Glu Glu Asp Glu Glu Glu Glu Glu Glu END
```

FIGURE 2: Nucleotide sequence and derived amino acid sequence of clone MK 2.1. Outlined on the top of the sequence is a partial restriction map and the sequencing strategy used. The lightly underlined sequences at the 5′ and 3′ ends of the clone represent *Eco*RI linkers. The boldly underlined sequences represent identity with peptide sequences obtained by Lukas et al. (1986) and Payne et al. (1986), respectively.

and digestion with S_1 nuclease, a 305-nucleotide piece was protected (Figure 3A). Thus, the first 55 bp of pMK 2.1 represent either a cloning artifact or a noncontiguous region of MLCK mRNA. In either case, the nonprotected fragment including the stop codon at nucleotides 40–42 is not relevant to further analysis of the remainder of pMK 2.1. The beginning of the MLCK cDNA is shown in Figure 2 to occur between nucleotides 55–60. The remainder of the cDNA is in a open reading frame until the termination codon at 2007.

The 0.35-kb cDNA did not hybridize to either pMK 1.0 or pMK 2.1 but did produce a fusion product that reacted with MLCK antibodies (Figure 5B). Therefore, the original library was rescreened with this insert in hopes of identifying a clone that would overlap pMK 0.35 and pMK 2.1. Of the positive

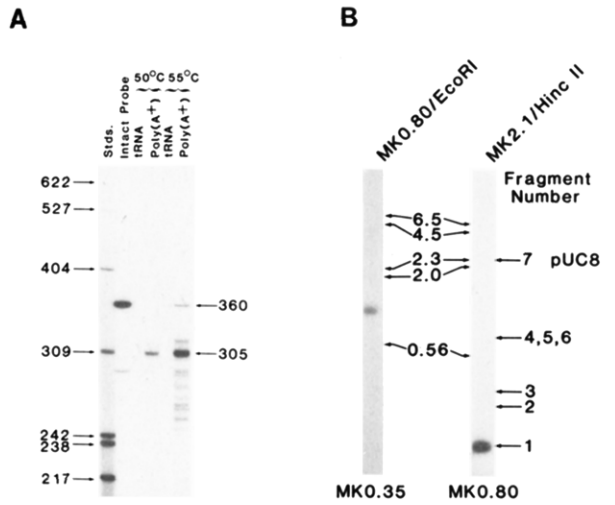


FIGURE 3: Evaluation of 5′ end of pMK 2.1. (A) S_1 protection. The 5′ 360-bp *Eco*RI–*Bgl*II fragment of pMK 2.1 was end-labeled and hybridized to either tRNA or gizzard poly(A+) RNA at the indicated temperatures. After digestion with S_1 nuclease, the fragments were separated by electrophoresis on a denaturing 5% acrylamide gel. The molecular weight standards on the left are end-labeled *Hpa*II fragments of pBR 322. The numbers on the right represent the size of the probe (360) and primary protected fragment (305), respectively. (B) Southern analysis. In the left panel, the insert of pMK 0.80 was electrophoresed on a 1% agarose gel, transfered to Biodyne A, and probed with the insert of pMK 0.35. In the right panel, pMK 2.1 was digested with *Hinc*II, and the fragments were separated by electrophoresis. After transfer to Biodyne, the filter was probed with the insert from pMK 0.80. The numbers in the middle represent λ DNA digested with *Hpa*II. The numbers on the right are the *Hinc*II fragments derived from pMK 2.1. They are ordered from 5′ (1) to 3′ (7) as shown at the top of Figure 7.

plaques obtained, one contained an 0.80-kb insert that was subloned into pUC 8. Figure 3B shows that the 0.80-kb cDNA hybridizes to both pMK 0.35 and the 5′ *Hinc*II fragment of pMK 2.1. This experiment suggested that pMK 0.80 might span the distance between pMK 0.35 and pMK 2.1. To examine this possibility, partial sequence of pMK 0.80 was obtained by the Maxam–Gilbert protocol. The plasmid was cut with *Eco*RI, 5′ end labeled with T_4 polynucleotide kinase, and recut with *Hin*fI. The 650-bp 5′ and 150-bp 3′ cDNA fragments were then subjected to sequence analysis. The only region of homology, on either strand, between these sequences and pMK 2.1 corresponds to the 5′ 53 bp of pMK 2.1. Since these nucleotides are precisely those not protected in the S_1 protection experiment shown in Figure 3A, pMK 0.80 and pMK 2.1 cannot directly overlap. Since pMK 0.8 presumably contains the 53 bp in its proper context, it is possible that these nucleotides were added to the 5′ end of pMK 2.1 during construction of the cDNA library. A looped-out secondary structure in the MLCK mRNA during the first strand synthesis or the cDNA during the second strand synthesis could have resulted in this artifactual association.

To further prove that pMK 2.1 contained MLCK cDNA, the derived amino acid sequence was compared to the amino acid sequence around one of the phosphorylation sites as determined by Payne et al. (1986). The 36 amino acids from the protein sequence match the derived amino acids from the nucleotide sequence (underlined in Figure 2). Also, Lukas et al. (1986) have recently reported a 20 amino acid sequence from the chicken gizzard MLCK that matches amino acids 493–512 (Figure 2) of the derived sequence. This sequence, which is reported to include the second phosphorylation site, is also underlined in Figure 2.

The derived amino acid sequence of the unique open reading frame was used to search a protein data bank for homologies. Two other protein kinases, the catalytic subunit of phosphorylase *b* kinase and cGMP-dependent protein kinase, were found to contain a high degree of homology (35%) to the chicken gizzard MLCK over a region of approximately 240 amino acids (Figure 4). This region corresponds to the catalytic domains of these two proteins (Takio et al., 1984). There was also a striking degree of homology (54%) in the same region when compared to the recently reported sequence of the carboxy terminus of rabbit skeletal muscle MLCK (Takio et al., 1985). Protein kinases contain a consensus sequence, Gly-Xxx-Gly-Xxx-Xxx-Gly, that is located 16–28 residues to the amino-terminal side of the lysine involved in ATP binding (Kamps et al., 1984). The region of homology that these three kinases share with chicken gizzard MLCK starts at or near this consensus sequence (Figures 2 and 4, amino acids 223–228) and extends 244–248 amino acids toward the carboxy terminus. Other proteins that show a high degree of homology include cell division control protein 28 from bakers' yeast (37% homology from amino acids 303–416) and the kinase-related transforming proteins (*mos*) from Muloney murine sarcoma virus (33% homology, amino acids 308–400), rat (36% homology, amino acids 308–400), and human (40% homology, amino acids 309–365). All of these homologies are within the catalytic domain of the kinase.

The calmodulin-binding region of the rabbit skeletal muscle MLCK is located at the carboxy terminus of the molecule (Blumenthal et al., 1985). Similarly, Foyt et al. (1985) suggested that the calmodulin-binding region of the chicken gizzard MLCK will lie to the carboxy terminus of the catalytic domain. In order to locate the calmodulin-binding domain in chicken smooth muscle MLCK, the plaque-purified phage were used to produce lysates from induced recombinant lysogens. As illustrated in Figure 5B, clones MK 2.1 and MK 1.0 produced proteins that were recognized by the antibody but were not represented as a fusion product with β-galactosidase. The β-galactosidase reading frame is not the same as the open reading frame of the cDNA insert. It is likely that the smaller peptides represent reinitiation of translation and/or protein degradation. All of the peptides represented in MK 1.0 are also present in MK 2.1. In addition, other peptides are seen in MK 2.1. Since the sequence of MK 1.0 is identical with the carboxy-terminal 1.0 kb of MK 2.1, the means used to generate the fragments must be identical. The products produced by clones MK 0.35 and MK 0.10 (Figure 5A) were fusion products as demonstrated by protein staining of the gel. The fusion product produced by clone MK 0.35 reacted with the antibody (Figure 5B), whereas the protein produced by cloned MK 0.10 did not react with the antibody (Figure 5B,). A possible explanation for this lack of reactivity may be due to the sample preparation and analysis using the Western transfer procedure since this clone reacted with the antibody during the screening procedure and could be used to purify a subset of antibodies from the polyclonal IgG (Figure 1).

Peptides produced by both MK 2.1 and MK 1.0 bound calmodulin in a calcium-dependent manner (Figure 5C,D). Once again, the patterns of the peptides were similar in that all of the calmodulin-binding peptides produced by MK 1.0 were also represented in those derived from MK 2.1. Clones MK 0.35 and MK 0.10 did not encode proteins that bind calmodulin. It was concluded that the region of MK 2.1 encoding the calmodulin-binding domain of MLCK was located in the 3′ half of the cDNA, the region that it shared in common with MK 1.0.

To more precisely define the calmodulin-binding region, the area of overlap was divided into different regions and subcloned

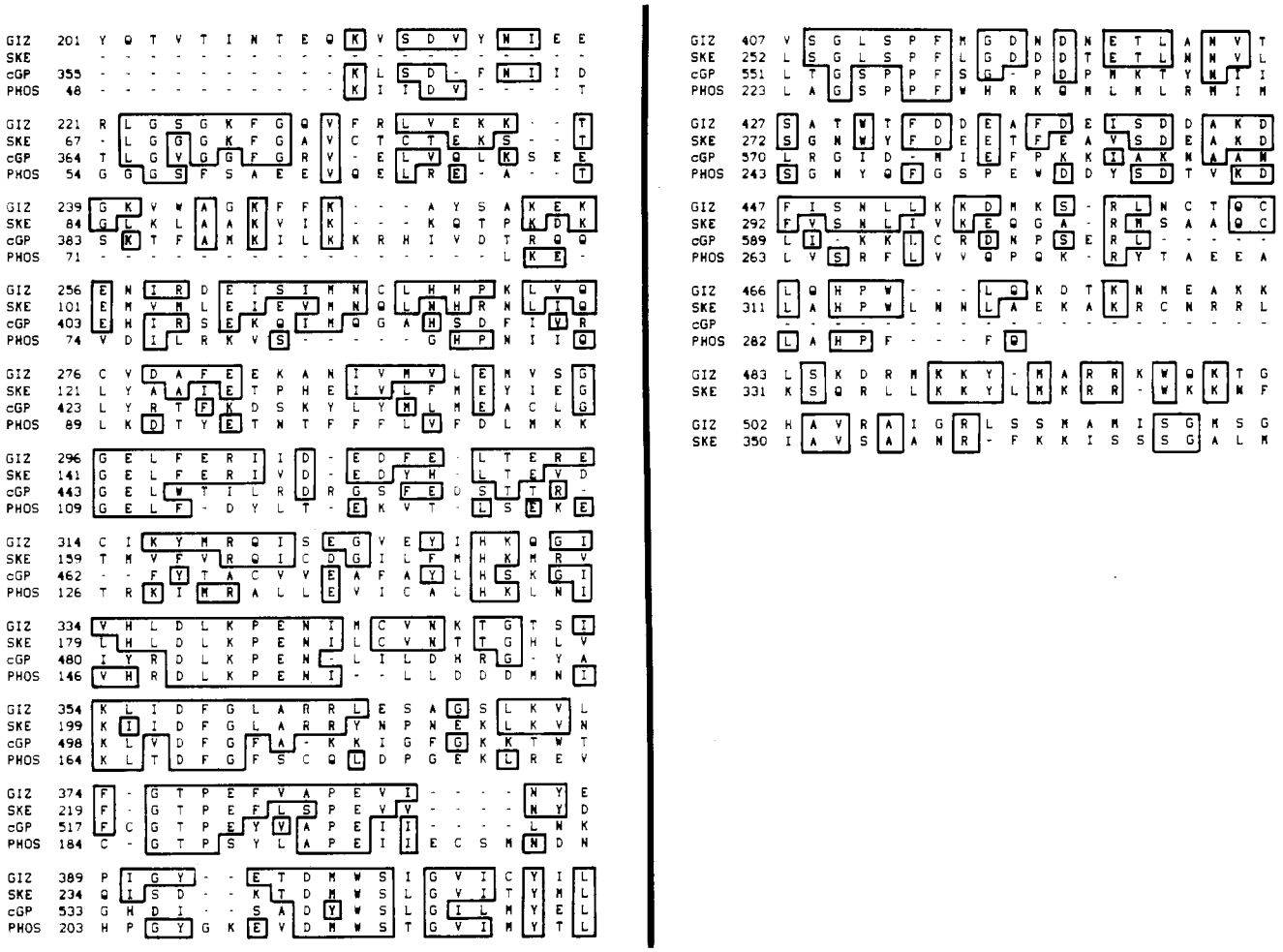


FIGURE 4: Alignment of a region of gizzard MLCK (GIZ) with rabbit skeletal MLCK (SKE), cyclic GMP dependent protein kinase (cGP), and the catalytic subunit of phosphorylase *b* kinase (PHOS). The boxed-in areas are direct amino acid homologies. Protein sequences were from the following references: rabbit skeletal muscle MLCK, Takio et al. (1985); cyclic GMP dependent protein kinase, Takio et al. (1985); catalytic subunit of phosphorylase *b* kinase, Reimann et al. (1984).

into the plasmid expression vector pUC19T (see Materials and Methods). The top of Figure 6 is a diagram showing the three different constructs that were tested. In all cases, those constructs in the open reading frame shown in Figure 2 produced protein products. The lysates from bacteria containing these inserts were analyzed for antibody binding (Figure 6A) and the ability to bind calmodulin in the presence (Figure 6B) and the absence (Figure 6C) of calcium. In both portions of the figure, an arrow designates the size of the peptide predicted from the amino acid sequence derived from the cDNA insert. All three constructs produced peptides that reacted with the antibody. Whereas pMK 470 and pMK 770 showed multiple bands indicating degradation of the expressed proteins, the antibody reactivity to the single pMK 540 peptide was weak. As indicated in Figure 5B,C, peptides derived from pMK 770 and pMK 540 bound calmodulin in a calcium-dependent manner whereas those from pMK 470 did not. Several other constructs containing cDNAs that produce peptides from proteins that do not bind calmodulin in a Ca^{2+}-dependent manner (ovalbumin, ovomucoid, and casein) did not react with ^{125}I-labeled calmodulin under the conditions used in this experiment (data not shown). Together these data suggest that the overlap between pMK 770 and pMK 540 (amino acids 463–526, Figure 2) must be the region that codes for the calmodulin-binding activity.

Figure 7A illustrates that all three clones (pMK 0.35, pMK 0.80, and pMK 2.1) recognize a 5.5-kb mRNA present in gizzard poly(A+) RNA. In vitro translation experiments on RNAs derived from a methylmercury hydroxide gel reveal that the mRNA encoding gizzard MLCK is about 5.5 kb long (data not shown). It can be seen from Figure 7A that pMK 2.1 also recognized a second mRNA species that is 2.7 kb in length. The minimum length of cDNA needed to code for a protein the size of MLCK (130 000 daltons) would be 3.4 kb; therefore, the smaller band cannot represent a mRNA for MLCK. As illustrated in Figure 7B, MK 2.1 was divided into seven fragments, and these were used to probe seven identical Northern blots. Probes one to four hybridized only to the larger mRNA, whereas probes five to seven hybridized to both mRNAs. These results indicate that the sequence 5′ to probe five is unique to the larger mRNA but the sequence contained in probes five to seven is shared by both mRNAs.

A series of S_1 nuclease protection and mapping experiments were done as a more stringent test for homology and to determine more precisely where the homology between the two mRNAs diverged. First, the entire MK 2.1 cDNA was used to hybridize to poly(A+) RNA followed by S_1 nuclease digestion. This would determine if there were small gaps of nonhomology that would not have been detected in the Northern blot experiments. The results show (Figure 8A) that most of MK 2.1 is protected as well as a smaller fragment of approximately 520 nucleotides. The large protected fragment corresponds to hybridization to the 5.5-kb mRNA, and the smaller fragment represents the region the 2.7-kb mRNA shares in common with the larger message. Also, there are no large gaps in the region of homology shared between the

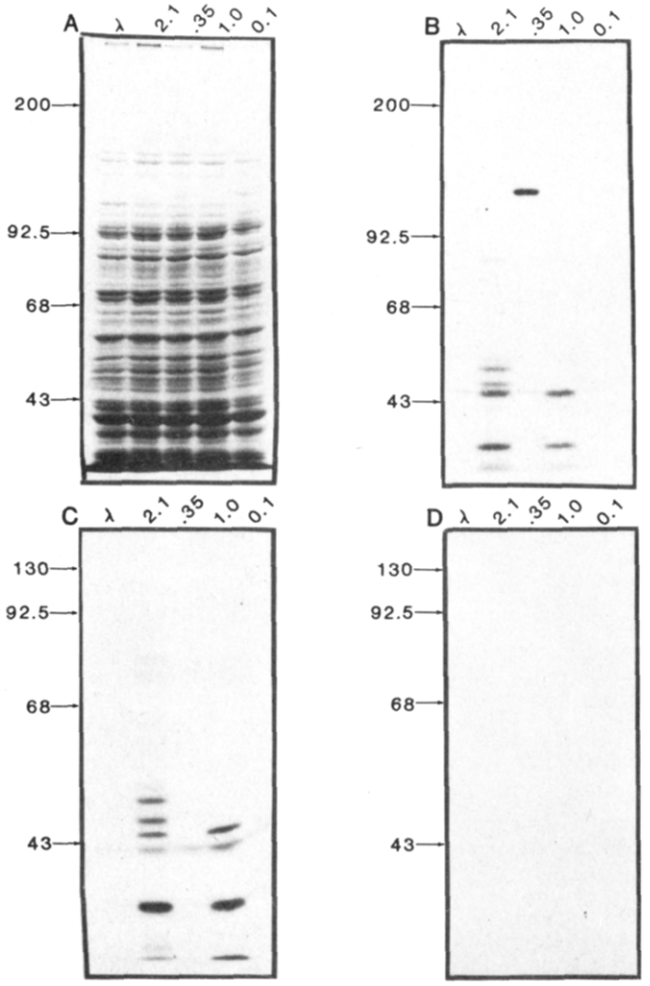


FIGURE 5: Analysis of fusion products. Bacteria were infected with phage, and fusion proteins were induced. Samples were electrophoresed on 7.5% polyacrylamide gels and analyzed by staining with Coomassie Blue G for protein (panel A), transferred to nitrocellulose and probed with anti-MLCK antibody (panel B), or assayed for ^{125}I-labeled calmodulin binding in the presence of Ca^{2+} (panel C) or EGTA (panel D). Extracts were made from bacteria infected with the following recombinant phage: λgt11 with no insert (lane λ), MK 2.1, MK 0.35, MK 1.0, and MK 0.10.

two mRNAs since only two protected fragments of the appropriate size are present.

An end-labeled probe was prepared that extended from nucleotide 1375 to nucleotide 1741 (*Hin*fI–*Taq*I fragment). The noncoding strand was labeled at position 1741, which corresponds to the 5′ overhang created by the *Taq*I site. This 366-nucleotide probe was hybridized to poly(A+) RNA followed by digestion with S_1 nuclease. The nuclease-resistant products were analyzed on a denaturing polyacrylamide gel (Figure 8B). Again, there were two resistant products, one at approximately 366 nucleotides corresponding to total probe protection. In this case, there are some slightly smaller bands that probably are a result of breathing at the end of the heteroduplex. The smaller much more abundant band migrated just ahead of the 242-nucleotide standard and from a linear regression analysis of the standards is 244 nucleotides in length. The region of divergence, then, is 244 nucleotides 5′ to the end-labeled *Taq*I site (nucleotide 1741) or at nucleotide 1497.

Discussion

We have described the isolation of several cDNA clones for chicken gizzard MLCK. The largest of these clones was

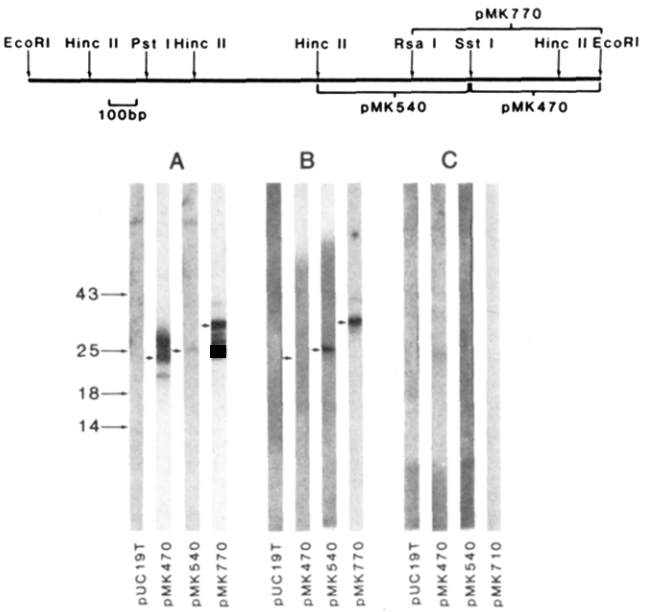


FIGURE 6: Analysis of expressed subclones. The top diagram shows the three regions that were inserted into the expression vector pUC19T. All were in the same frame as that shown as the open reading frame in Figure 2. Cultures of each of these subclones were grown overnight, and the samples were electrophoresed on polyacrylamide gels, transferred to nitrocellulose paper, and analyzed for anti-MLCK antibody reactivity (A) on ^{125}I-labeled calmodulin binding in the presence of Ca^{2+} (B) or EGTA (C). The arrows present the primary peptide synthesized in each instance. The molecular size markers on the left of the figure are in kilobases.

sequenced and contains the coding region for approximately 60% of the enzyme including the carboxy terminus. Authenticity of this clone was demonstrated by several independent criteria that include (1) the ability of clone purified antibody to recognize a protein in total homogenates that comigrates with MLCK, (2) the demonstration of calmodulin-binding activity by proteins produced by the clone in bacteria, (3) the direct amino acid homology (54%) with rabbit skeletal muscle MLCK, and (4) the amino acid sequence confirmation.

A model for chicken gizzard MLCK was derived from the information presented in this paper (Figure 9). The relative placements of the catalytic domain, calmodulin-binding domain, and one of the phosphorylation sites are all consistent with the model presented by Foyt et al. (1985). The 20 amino acid calmodulin-binding peptide from chicken gizzard MLCK indentified by Lukas et al. (1986) is located in the region (amino acids 493–512, Figure 2) we have defined as the calmodulin-binding domain. This peptide contains the calmodulin-regulated phosphorylation site, which is also consistent with the model of Foyt et al. (1985). Furthermore, the rabbit skeletal muscle form of the enzyme has a similar arrangement of the catalytic and calmodulin-binding domains (Edelman et al., 1985). A major difference between these two enzymes is that the calmodulin-binding region of the rabbit skeletal muscle enzyme is located at the carboxy terminus whereas in the gizzard form of the enzyme there are approximately 142 amino acids carboxy terminal to the calmodulin binding domain. Another unusual feature of the gizzard enzyme is the high number of acidic residues (amino acids 653–669) near the carboxy terminus. To more clearly define the calmodulin-binding domain of the smooth muscle enzyme, smaller peptides have been chemically synthesized in collaboration with Dr. Bruce Kemp at the University of Melbourne. These studies further restrict the calmodulin-binding region to amino acids 480–510 and reveal that this fragment also acts as a

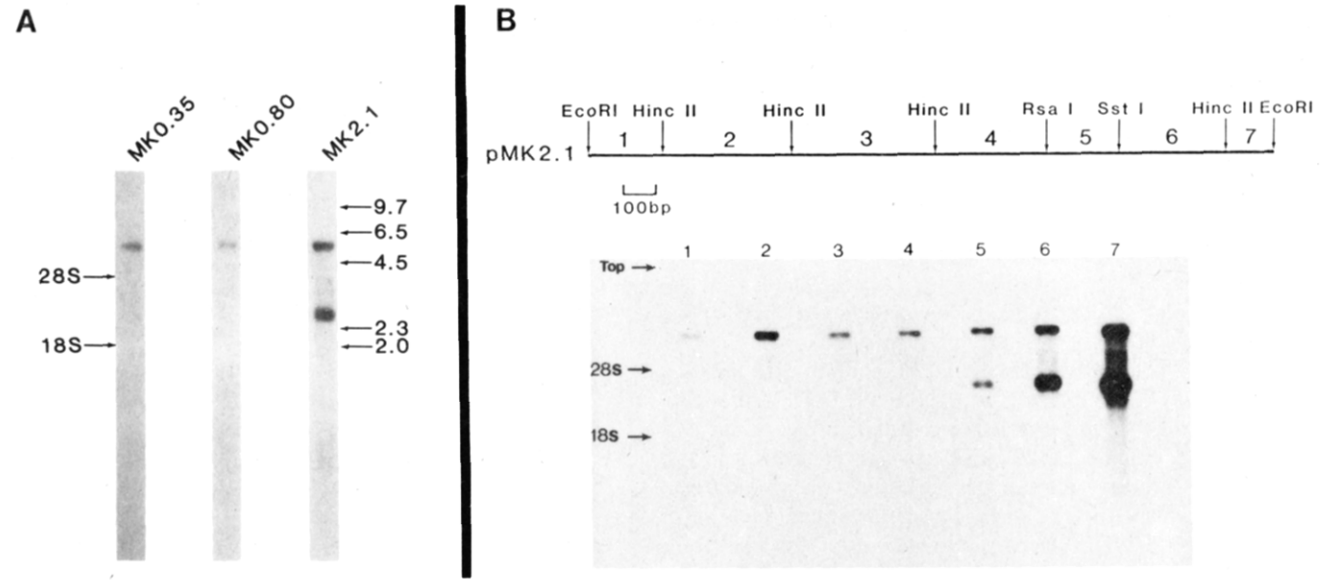


FIGURE 7: Hybridization of chicken gizzard poly(A+) RNA to various MLCK cDNA clones. (A) Identical blots of chicken gizzard poly(A+) RNA were hybridized with the entire inserts from MK 0.35, MK 0.80, or MK 2.1. Migration of the 28S and 18S ribosomal RNAs are indicated on the left whereas the right indicates DNA size markers. In all cases the numbers are in kilobases. (B) Seven identical Northern blots were probed with fragments derived from the MK 2.1 cDNA insert. The numbers represent the fragments illustrated in the partial restriction map of MK 2.1 shown at the top of the figure.

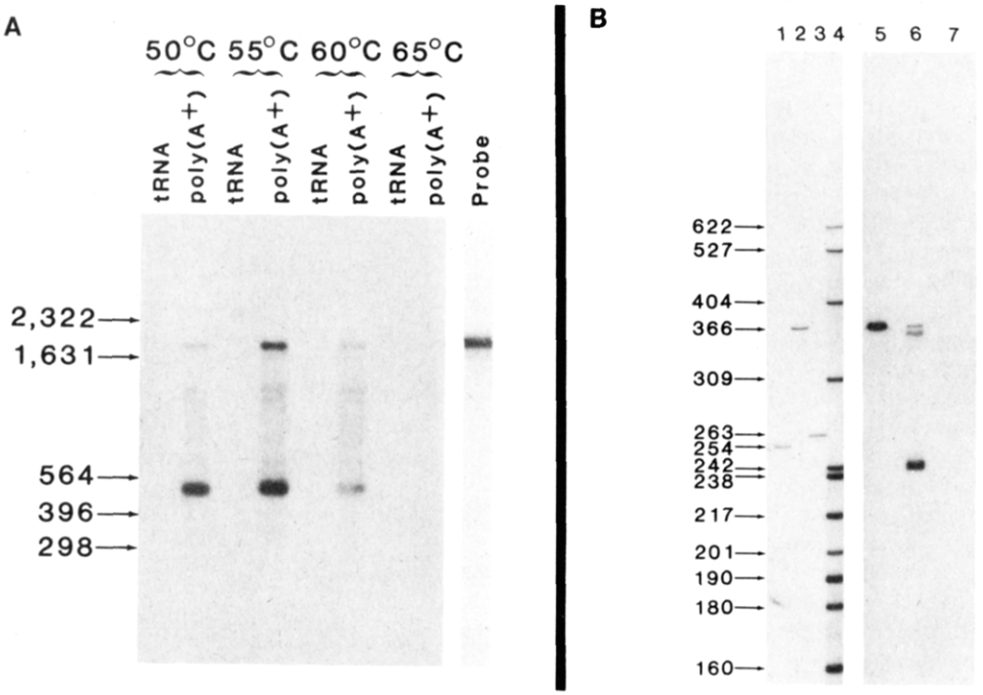


FIGURE 8: S_1 protection and mapping. (A) Entire MK 2.1 was hybridized to either poly(A+) RNA or tRNA (at the specified temperatures), treated with S_1 nuclease, electrophoresed on a nondenaturing agarose gel, transferred to Biodyne A membrane, and probed with MK 2.1. Numbers on the right are the sizes (in base pairs) of standards. (B) An end-labeled probe (nucleotides 1375–1741) was hybridized to poly(A+) RNA or tRNA and treated with S_1 nuclease, and samples were electrophoresed on a 5% denaturing polyacrylamide gel. Lanes 1–4 contain molecular weight markers of the indicated sizes (base pairs), lane 5 was probed with no RNA but the sample went through the entire procedure except for S_1 nuclease treatment, and in lane 6 the probe was incubated with poly(A+) RNA whereas in lane 7 the probe was incubated with tRNA.

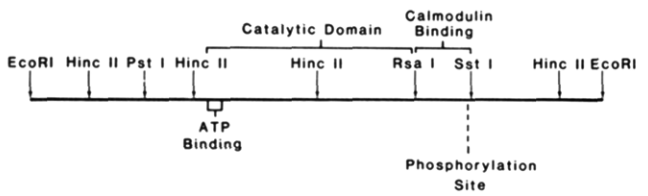


FIGURE 9: Organization of functional domains of chicken gizzard MLCK derived from information provided in this paper.

substrate antagonist of chicken gizzard myosin light chain kinase (Kemp et al., 1987).

The rabbit skeletal muscle enzyme and the chicken gizzard enzyme have highly homologous catalytic domains (54% direct amino acid homology), and no gaps are needed to maximize identity over 248 amino acids (amino acids 222–470). Amino acids 342–360 of skeletal muscle MLCK contain the calmodulin-binding activity (Edelman et al., 1985). The gizzard form of the enzyme displays a 38% homology in this region (Figure 4, amino acids 493–513), but two gaps are needed for the alignment. This region falls within the area we have defined as containing the calmodulin-binding activity. Finally, on the amino-terminal side of the rabbit skeletal muscle

calmodulin-binding region is the sequence Lys-Lys-Tyr (amino acids 437–439), which is identical in the gizzard sequence (amino acids 489–491). It will be of interest to determine if this tripeptide is involved in calmodulin binding by the skeletal muscle enzyme.

In the calmodulin-binding region (amino acids 463–526) there is a high number of basic residues (lysine and arginine) that can serve as cleavage sites for trypsin. This is consistent with the finding that the calmodulin dependency of MLCK is extremely sensitive to trypsin cleavage (Tanaka et al., 1980; Foyt et al., 1985). Other calmodulin-dependent enzymes such as the catalytic subunit of phosphorylase *b* kinase (Cohen, 1973; DePaoli-Roach et al., 1979) and calmodulin-dependent cyclic nucleotide phosphodiesterase (Moss et al., 1978; Epstein et al., 1978) can also be converted to calmodulin-independent forms by proteolytic degradation. It has been suggested that the carboxy terminus of the subunit of phosphorylase *b* kinase is the portion of the molecule that interacts with calmodulin (Reimann et al., 1984). The carboxy terminus of this enzyme does contain clusters of basic residues, which is consistent with this feature for calmodulin-binding domains.

The results from the Northern blots and S_1 protection experiments demonstrate the presence of another chicken gizzard mRNA (2.7 kb) that shares a high degree of homology with or is identical with approximately 520 nucleotides at the 3′ end of MK 2.1. The labeled probe used for the S_1 mapping experiments was the noncoding strand. Therefore, the homology cannot exist with the noncoding strand of MLCK. These data raise the possibility that the proteins encoded by these two mRNAs share a common functional domain. The region of divergence occurs toward the end of the domain that contains the calmodulin-binding activity of MLCK. Kemp et al. (1987) have determined that a peptide containing amino acids 480–501 both is a strong calmodulin antagonist and acts as a MLCK pseudosubstrate. All of the amino acids present in this peptide lie within the region that is unique to MLCK and not shared by the two mRNAs. Further toward the carboxy terminus is a serine (amino acid 511 or 512) that Lukas et al. (1986) have suggested to be the calmodulin-regulated phosphorylation site of MLCK, and further still is another serine (amino acid 525) that Payne et al. (1986) have shown to be phosphorylated by cyclic AMP dependent protein kinase. Both of these phosphorylation sites are contained in the region of shared homology. Efforts are now being made to isolate cDNA clones for the smaller second mRNA as well as full-length cDNA for MLCK. The sequence of cDNAs for the smaller mRNA will determine the exact degree of shared homology, confirm where the divergence occurs, and aid in assigning a function by amino acid sequence homologies with other known proteins.

ACKNOWLEDGMENTS

We express our appreciation to Dr. Paul Epstein for helpful discussions and Charles R. Mena for his skillful technical assistance.

Registry No. ATP, 56-65-5; MLCK, 51845-53-5; phosphorylase *b* kinase, 9001-88-1; protein kinase, 9026-43-1.

REFERENCES

Adelstein, R. S., & Klee, C. B. (1980) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. 1, pp 167–182, Academic, New York.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, L., Walsh, K. A., & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3187–3191.

Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J. 133*, 529–539.

Chafouleas, J. G., Dedman, J. R., Munjaal, R. P., & Means, A. R. (1979) *J. Biol. Chem. 254*, 10262–10267.

Cohen, P. A. (1973) *Eur. J. Biochem. 34*, 1–14.

Conti, M. A., & Adelstein, R. S. (1981) *J. Biol. Chem. 256*, 3178–3181.

DePaoli-Roach, A. A., Roach, P. J., & Larner, J. (1979) *J. Biol. Chem. 254*, 4212–4219.

Edelman, A. M., & Krebs, E. G. (1982) *FEBS Lett. 138*, 293–298.

Epstein, P. M., Pledger, W. J., Gardner, E. A., Stancel, G. M., Thompson, W. J., & Strada, S. J. (1978) *Biochim. Biophys. Acta 527*, 442–455.

Feinberg, A. P., & Volgelstein, B. (1983) *Anal. Biochem. 132*, 6–13.

Foyt, H. L., Guerriero, V., Jr., & Means, A. R. (1985) *J. Biol. Chem. 260*, 7765–7774.

Gubler, H., & Hoffman, B. (1983) *Gene 25*, 263–269.

Guerriero, V., Rowley, D. R., & Means, A. R. (1981) *Cell (Cambridge, Mass.) 27*, 449–458.

Hanahan, D. (1983) *J. Mol. Biol. 16*, 557–580.

Kamps, M. P., Taylor, S. S., & Sefton, B. M. (1984) *Nature (London) 310*, 389–391.

Kemp, B. E., Pearson, R. B., Guerriero, V., Jr., Bagchi, I. C., & Means, A. R. (1987) *J. Biol. Chem.* (in press).

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lukas, T. J., Burgess, W. H., Prendergas, F. G., Lau, W., & Watterson, D. M. (1986) *Biochemistry 25*, 1458–1464.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maxim, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Moss, J., Manganiello, V. C., & Vaughan, M. (1978) *Biochim. Biophys. Acta 541*, 270–287.

Nishikawa, M., de Lanerolle, P., Lincoln, T. M., & Adelstein, R. S. (1984) *J. Biol. Chem. 259*, 8429–8436.

Payne, M. E., Elzinga, M., & Adelstein, R. S. (1986) *J. Biol. Chem.* (in press).

Queen, C., & Korn, L. J. (1984) *Nucleic Acids Res. 12*, 581–599.

Reimann, E. M., Titani, K., Ericsson, L. H., Wade, R. D., Fisher, E. H., & Walsh, K. A. (1984) *Biochemistry 23*, 4185–4192.

Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., & O'Malley, B. W. (1975) *Biochemistry 14*, 69–78.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Sellers, J. R., & Harvey, E. V. (1984) *Biochemistry 23*, 5831–5836.

Southern, E. (1975) *J. Mol. Biol. 98*, 503.

Takio, K., Wade, R. D., Smith, S. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1984) *Biochemistry 23*, 4207–4218.

Takio, K., Blumenthal, D. K., Edelman, A. M., Walsh, K. A., Krebs, E. G., & Titani, K. (1985) *Biochemistry 24*, 6028–6037.

Tanaka, T., Naka, M., & Hidaka, H. (1980) *Biochem. Biophys. Res. Commun. 92*, 313–318.
Young, R. A., & Davis, R. W. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194–1198.
Young, R. A., & Davis, R. W. (1983b) *Science (Washington, D.C.) 222*, 778–782.
Wolf, H., & Hofmann, F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5852–5855.

Structural and Functional Properties of a Phospholipase A2 Purified from an Inflammatory Exudate†

Steven Forst, Jerrold Weiss, and Peter Elsbach*
Department of Medicine, New York University Medical Center, New York, New York 10016
John M. Maraganore,‡ Ilene Reardon, and Robert L. Heinrikson
Biopolymer Chemistry Unit, The Upjohn Company, Kalamazoo, Michigan 49001
Received September 5, 1986; Revised Manuscript Received October 8, 1986

ABSTRACT: The cell-free supernatant of sterile inflammatory peritoneal exudates contains a phospholipase A2 that participates in the digestion of *Escherichia coli* killed by polymorphonuclear leukocytes or by the purified bactericidal/permeability increasing protein (BPI) of these cells. This phospholipase A2 has been purified, and the sequence of the NH_2-terminal 39 amino acids has been determined and compared with sequences of both BPI-responsive and BPI-nonresponsive phospholipases A2 from snake venoms and mammalian pancreas. The high concentration and location of basic residues in the NH_2-terminal region is a common feature of BPI-responsive phospholipases A2 and may characterize those phospholipases A2 participating in inflammatory events.

Phospholipases such as phospholipase A2 (PLA2)[1] are thought to be involved in inflammation (Vadas & Pruzanski, 1984; Ahnfeld-Ronne & Arrigoni-Martelli, 1984; Flower, 1984). The search for means of altering phospholipase action in the host in order to modify inflammatory responses would be helped greatly by precise information about the phopsholipases involved in specific biological events. Whereas much is known about the structure and function of secretory PLA2 (Heinrikson, 1982; Dennis, 1983; Verheij et al., 1981), little is known about the phospholipases acting on the phospholipids of inflammatory cells and their targets. Most of these phospholipases are cellular enzymes that are present in trace amounts and therefore not readily available for detailed study.

Our studies on PLA, particularly PLA2, have dealt with the determinants of bacterial phospholipid degradation in relation to host defense against infection (Elsbach et al., 1979, 1985; Weiss et al., 1978, 1979; Elsbach & Weiss, 1983; Forst et al., 1982, 1986a). The antibacterial action of polymorphonuclear leukocytes (PMN) is accompanied by bacterial phospholipid degradation in which bacterial and PMN PLA participate. Using *Escherichia coli* as a test microorganism, we have found that in the PMN the activating agent for PLA's that degrade the bacterial phospholipids is a potent membrane-active and bactericidal protein that is specific for Gram-negative bacteria (Elsbach et al., 1979; Weiss et al., 1978, 1979; Elsbach & Weiss, 1983; Forst et al., 1982). The activation of PLA by this bactericidal/permeability-increasing protein (BPI) is highly selective. Of the 14 purified PLA2 that have been tested, only the PMN PLA2 and two basic snake venom enzymes can degrade the phospholipids of BPI-killed *E. coli* (Weiss et al., 1979; Forst et al., 1982; Elsbach et al., 1986). Chemical modification and primary structural analyses of the latter two enzymes have revealed a cluster of basic residues in the NH_2-terminal 15 amino acid segment that appears to be important in their action toward BPI-killed *E. coli* (Forst et al., 1986a; Elsbach et al., 1986). The scarcity of the PMN PLA2, however, has precluded similar analysis of the PLA2 of this inflammatory cell (Elsbach et al., 1979).

A much richer source of "inflammatory" PLA2 is the cell-free supernatant (ascitic fluid) of the PMN-containing sterile inflammatory exudates that can be elicited in the peritoneal cavity of rabbits (Franson et al., 1978). Added ascitic fluid can markedly enhance the degradation of phospholipids of *E. coli* killed by the PMN (Forst et al., 1986b), which suggests that its PLA2 can also contribute to bacterial phospholipid degradation. We have now purified the ascitic fluid PLA2 to homogeneity, which permits structural and functional comparison between this inflammatory exudate enzyme and BPI-responsive and -unresponsive PLA2's that have been studied previously.

Materials and Methods

Collection of Ascitic Fluid. Sterile inflammatory peritoneal exudates were collected 14–18 h after intraperitoneal injection

† This study was supported by U.S. Public Health Service Grants AI 18571 and AM 05472.
* Author to whom correspondence should be addressed.
‡ Present address: Zymogenetics, Inc., Seattle, WA 98103.

[1] Abbreviations: BPI, bactericidal permeability increasing protein; PLA2, phospholipase A2; PMN, polymorphonuclear leukocyte; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Gdn-HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

0006-2960/86/0425-8381$01.50/0 © 1986 American Chemical Society