MOLECULAR CLONING AND CHARACTERIZATION OF A cDNA FOR BOVINE PHOSPHOLIPASE C-α: PROPOSAL OF REDESIGNATION OF PHOSPHOLIPASE C-α[1]

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We have isolated bovine phospholipase C (PLC)-α cDNA from bovine thymus. Sequence analysis showed that PLC-α is highly conserved among rat, mouse, and calf and that it has two Trp-Cys-Gly-His-Cys-Lys motifs completely conserved in the mammals. Southern blot analysis revealed that bovine PLC-α is derived from a single gene. When PLC-α cDNA was stably transfected in NIH3T3 cells, there was no increase in PLC activity. PLC-α is supposed to be a member not of PLC superfamily but of Trp-Cys-Gly-His-Cys-Lys motif-containing proteins consisting of protein disulfide isomerase, P5, ERp72, and thioredoxin. PLC-α should be redesignated ERp57 (ER-resided p57).

Phospholipase C (PLC) has been shown to be a superfamily composed of different isozymes, and cDNA clones encoding nine different PLC isoforms have been cloned [1-7]. Of the members, however, PLC-α is a notable exception since PLC-α fails to share significant sequence similarity with any of the known PLCs

[1]The nucleotide sequence data reported in this paper will appear in the DDBJ/GenBank/EMBL Nucleotide Sequence Database with accession number D16234.

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and does not contain PLC enzymatic domain or calcium-binding domain. Instead, PLC-α contains a characteristic 110 amino-acid sequence which is repeated twice within the protein sequence. In each repeat there exists a Trp-Cys-Gly-His-Cys-Lys motif, which is identical to the active sites of protein disulfide isomerase [8], P5 [9], and ERp72 [10] and highly similar to those of thioredoxin [11].

So far rat and mouse PLC-α cDNAs have been cloned. Here we report the isolation and characterization of a cDNA coding for bovine PLC-α. Moreover, we established the stable transfectants overexpressing PLC-α and show that PLC-α cDNA does not encode any functional PLC activity.

MATERIALS AND METHODS

Cloning of bovine PLC-α cDNA A total of 1.8×10^6 plaques from a λgt11 bovine thymus cDNA expression library were plated in the bacterial strain Y1090R$^-$. Plates were incubated for 3.5 h at 43°C and then β-galactosidase fusion protein expression was induced by overlaying the lawns with isopropylthio-β-D-galactosidase (10 mM)-impregnated nitrocellulose filters (Hybond C-Extra). Transfer of released proteins was allowed to proceed 3 h at 37°C. Filters were blocked with 1% bovine serum albumin (Fraction V) in TBST buffer (10 mM Tris-HCl (pH7.0), 150 mM NaCl, 0.05% Tween20). Partial PLC-α cDNA clones were identified by probing the filters with 507 antiserum. To further obtain the 5' part of the cDNA, the same library was screened using partial PLC-α cDNA clones labeled with [^{32}P] dCTP by the random hexanucleotide random extension method (Amersham). PLC-α cDNA clones were subcloned into the *Eco*RI site of pBluescript SK(-) (Stratagene). Nested deletions were generated using the Exo-Mung deletion kit (Pharmacia). Double-stranded DNA sequencing was carried out using the Sequenase 2.0 system (USB).

Southern blot analysis Genomic DNA of bovine thymus was digested with restriction enzymes, and restriction fragments were analyzed by Southern filter hybridization with the radiolabeled full length bovine PLC-α cDNA. The filter was washed in 0.1 × SSC and 0.1% sodium dodecyl sulfate at 65°C.

Establishment of stable transfectants The full length bovine PLC-α cDNA was inserted into downstream of β-actin promoter of pUC-CAGGS, a mammalian expression vector, generating PLC-α sense and antisense constructs. The PLC-α expression plasmids were transfected into NIH3T3 fibroblasts by calcium phosphate coprecipitation along with pSV2NEO and selected with G418 (800 μg/ml; total drug). Emergent colonies were grown up to produce clonal cell lines (F01, F02, and F03 transfected with a sense construct; R02 and R06 transfected with an antisense construct).

Immunoblotting Cells were grown up to confluent state, and lysed on ice in RIPA lysis buffer, 50 mM Tris-HCl (pH7.4), 150 mM NaCl, 0.05% (w/v) sodium dodecyl sulfate, 1% sodium deoxycholate, 1% (v/v) Triton X-100, 10 U/ml aprotinin, 2 mM phenylmethylsulfinyl fluoride, 100 mg/l leupeptin, 1 mM sodium orthovanadate. Insoluble materials were removed by centrifugation. Protein concentration was determined with a protein assay kit (Bio-Rad) and equal amounts of cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred to Immobilon-P membrane filters (Millipore). The filters were blocked for 3 h in TBST buffer containing 1% bovine serum albumin (Fraction V) and incubated for 1 h with 507 antiserum in TBST buffer. The filters were then washed three times with TBST buffer and incubated with alkaline phosphatase-conjugated goat anti-rabbit second antibody for 1 h.

Measurement of PLC activities PLC activities in the homogenates, cytosol, and membrane fractions of the NIH3T3 sublines were measured with [^{3}H]-labeled phosphatidylinositol 4,5-P_2 as a substrate. Preparation of cells and measurement of PLC activity with [^{3}H]-labeled phosphatidylinositol 4,5-P_2 as a substrate were performed as described previously [12].

RESULTS AND DISCUSSION

This study was initially undertaken in an effort to clone a new SH2 (*src*-homology region 2)-containing molecule [13]. We raised a rabbit polyclonal antibody against Gly-Ser-Phe-Leu-Val-Arg-Glu-Ser peptide which is conserved among SH2 regions of various proteins. In bovine thymus, a 50 kDa protein was one of the major proteins recognized by this antibody. We purified this protein and raised a rabbit polyclonal antibody (named 507 antiserum). Unexpectedly, this antibody recognized a 57 kDa protein as well as a 50 kDa protein. A 50 kDa protein was later identified bovine homologue of *csk* [14], but the identity of the 57 kDa protein remained unknown. We screened a λgt11 bovine thymus cDNA expression library with 507 antiserum and obtained seven immunoreactive clones. Sequence analysis and homology search revealed that they encoded bovine homologue of PLC-α protein, but lacked the amino-terminal methionine. To further obtain the 5' part of the cDNA, the same library was screened using the most 5' end fragment of the partial PLC-α cDNA clones. The full length bovine PLC-α cDNA consists of a 1515-bp open-reading frame predicting a polypeptide of 505 amino acids with a calculated molecular mass of 56,895 Dalton. It contains polyadenylation signal

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   1  GGGTACGTGGTTGCCGGCCCGGTCTCCGCAGTCCCCACCGAGCCCCGACC
  51  CTCCTGGCCGCCCCCCTCGCCTTACCTCGTCGCCATGCGCCTCCGCCGCCT
                                          M  R  L  R  R  L
 101  AGCGCTGTTCCCAGGCCTGGCGCTGCTCCTCGCCGCGGCCCGCCTCGCTG
       A  L  F  P  G  L  A  L  L  L  A  A  A  R  L  A  A
 151  CTGCCTCCGATGTGCTGGAACTCACGGACGACAACTTCGAGAGTCGCATC
         A  S  D  V  L  E  L  T  D  D  N  F  E  S  R  I
 201  ACCGACACGGGCTCTTCTGGCCTCATGCTCGTCGAGTTCTTCGCCCCCTG
       T  D  T  G  S  S  G  L  M  L  V  E  F  F  A  P  W
 251  GTGTGGACACTGCAAAAAGCTTGCCCCAGAGTATGAAGCTGCAGCTACCA
         C  G  H  C  K  K  L  A  P  E  Y  E  A  A  A  T  R
 301  GATTAAAAGGAATAGTCCCGTTAGCAAAGGTGGATTGTACTGCCAACACG
         L  K  G  I  V  P  L  A  K  V  D  C  T  A  N  T
 351  AACACCTGTAATAAGTATGGAGTGAGTGGATATCCAACCCTGAAGATATT
       N  T  C  N  K  Y  G  V  S  G  Y  P  T  L  K  I  F
 401  TAGAGATGGTGAAGAATCAGGTGCCTATGATGGGCCTAGGACTGCCGATG
         R  D  G  E  E  S  G  A  Y  D  G  P  R  T  A  D  G
 451  GAATTGTCAGCCACCTGAAGAAACAGGCTGGACCAGCTTCAGTTCCTCTC
           I  V  S  H  L  K  K  Q  A  G  P  A  S  V  P  L
 501  AAGTCTGAGGAAGAATTTGAAAAGTTCATTAGCGATAAAGATGCTTCTGT
       K  S  E  E  E  F  E  K  F  I  S  D  K  D  A  S  V
 551  GGTGGGTTTTTTCAAGGATTTATTCAGTGAAGCTCACTCTGAGTTTCTAA
         V  G  F  F  K  D  L  F  S  E  A  H  S  E  F  L  K
 601  AAGCAGCCAGCAACTTGAGGGATAACTACCGGTTTGCACACACCAATGTT
         A  A  S  N  L  R  D  N  Y  R  F  A  H  T  N  V
 651  GAATCTCTGGTGAACAAATACGATGACGATGGAGAGGGTATCACCTTGTT
       E  S  L  V  N  K  Y  D  D  D  G  E  G  I  T  L  F
 701  TCGTCCTTCCCATCTGACGAACAAGTTTGAAGACAAGACTGTGGCATATA
         R  P  S  H  L  T  N  K  F  E  D  K  T  V  A  Y  T
 751  CAGAACAGAAAATGACCAGTGGGAAGATTAAAAGATTTATTCAGGAAAAC
           E  Q  K  M  T  S  G  K  I  K  R  F  I  Q  E  N
 801  ATTTTTGGTATCTGCCCTCACATGACAGAAGACAATAAAGATTTACTACA
       I  F  G  I  C  P  H  M  T  E  D  N  K  D  L  L  Q
 851  GGGCAAGGATTTACTCATCGCTTACTATGATGTGGACTATGAAAAGAATG
         G  K  D  L  L  I  A  Y  Y  D  V  D  Y  E  K  N  A
 901  CTAAAGGTTCCAACTACTGGAGAAACAGAGTAATGATGGTGGCAAAGAAA
           K  G  S  N  Y  W  R  N  R  V  M  M  V  A  K  K
 951  TTCCTGGATGCTGGGCAGAAACTCCATTTTGCTGTAGCTAGCCGTAAAAC
       F  L  D  A  G  Q  K  L  H  F  A  V  A  S  R  K  T
1001  CTTTAGCCATGAACTTTCAGATTTTGGCTTGGAAAGCACTACTCGAGAGA
         F  S  H  E  L  S  D  F  G  L  E  S  T  T  G  E  I
1051  TTCCTGTTGTTGCTGTCAGAACCGCAAAAGGAGAGAAGTTTGTCATGCAG
         P  V  V  A  V  R  T  A  K  G  E  K  F  V  M  Q
1101  GAGGAGTTCTCGCGTGATGGCAAGGCTCTTGAGAGATTCCTGGAGGATTA
       E  E  F  S  R  D  G  K  A  L  E  R  F  L  E  D  Y
1151  CTTTGACGGCAACCTGAAGAGATACCTGAAGTCTGAGCCTATCCCTGAGA
         F  D  G  N  L  K  R  Y  L  K  S  E  P  I  P  E  S
1201  GCAATCATCGGCCTGTAAAGGTAGTGGTAGCAGAGAATTTTGATGAAATA
           N  D  G  P  V  K  V  V  V  A  E  N  F  D  E  I
1251  GTGAATAATGAAAATAAAGATGTGCTGATTGAGTTTTATGCTCCTTGGTG
       V  N  N  E  N  K  D  V  L  I  E  F  Y  A  P  W  C
1301  TGGTCACTGTAAGAATCTGGAGCCTAAGTATAAAGAACTGGGAGAGAAGC
         G  H  C  K  N  L  E  P  K  Y  K  E  L  G  E  K  L
1351  TCAGAAAAGATCCAAATATTGTCATAGCCAAGATGGATGCTACAGCCAAC
           R  K  D  P  N  I  V  I  A  K  M  D  A  T  A  N
1401  GATGTGCCTTCTCCATATGAAGTCAGAGGTTTTCCTACCATCTACTTCTC
       D  V  P  S  P  Y  E  V  R  G  F  P  T  I  Y  F  S
1451  TCCAGCCAACAAGAAGCAAAATCCAAAGAAATATGAAGGTGGCCGTGAAT
         P  A  N  K  K  Q  N  P  K  K  Y  E  G  G  R  E  L
1501  TAAGTGATTTTATTAGCTATCTAAAGCGAGAGGCTACAAACCCCCCTGTA
           S  D  F  I  S  Y  L  K  R  E  A  T  N  P  P  V
1551  ATTCAAGAAGAAAAACCCAAGAAGAAGAAGAAGGCACAGGACGATCTCTA
       I  Q  E  E  K  P  K  K  K  K  K  A  Q  E  D  L
1601  AAGCAGCAGCCAAACATCATATACTTTGTCAAAGGACTTTTCCACCAGAG
1651  ATGGGAAAACCAATGGGGAGGACTGGGACCCGTATGGGAATTACTGCCTC
1701  TCAGGGCTGAGAGGGCAGAATGGTTATAATCTGAGTCCTGTTAAATTTTC
1751  TCTAAACTGTTTCTTAGCTGCACTGTTTATGAAAATACCAGAACCAGTTT
1801  ATGTTTGTGGTTTTGGGAAAATTATTTGTGTTGAGGGGAATGTTGTGGGG
1851  GTGGGGGGAATTGAGTTGGGGGGTTATTTTCTAATTTTTTTTGTACATTT
1901  GGAACAGTGACAATAAATGCGCCCCCTTTAAAAAAAAAAAAA
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Figure 1. The nucleotide and deduced amino acid sequences of bovine PLC-α. The deduced amino acid sequence is shown in one-letter code. Polyadenylation signal is underlined.

(AATAAA) 18 bp upstream of poly (A) tail and hydrophobic N-terminal signal peptides (amino acid residues 1-24) predicted by the Von Heijne method [15] (Fig. 1). The deduced amino acid sequence of bovine PLC-α exhibits an overall identity of 90% and 87% to those of murine [16] and rat [1] PLC-α, respectively. Interestingly, the sequences of the 3' untranslated region of PLC-α were very highly conserved among calf, mouse, and rat showing around 90% identity at the nucleotide level. This may imply the existence of *cis*-acting nucleic acid sequences with some important regulatory functions.

Southern blot analysis of bovine thymus DNA with a probe for bovine PLC-α cDNA is shown in Fig. 2. Single band is detected in digest with *Hind*III, and the locus is contained within a 8.5-kilobase *Hind*III fragment. This indicates that the bovine PLC-α gene is present as a single copy gene.

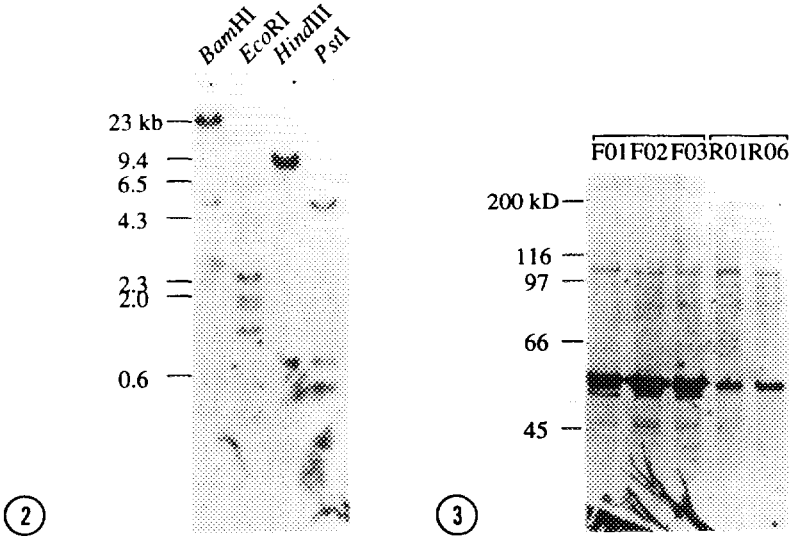
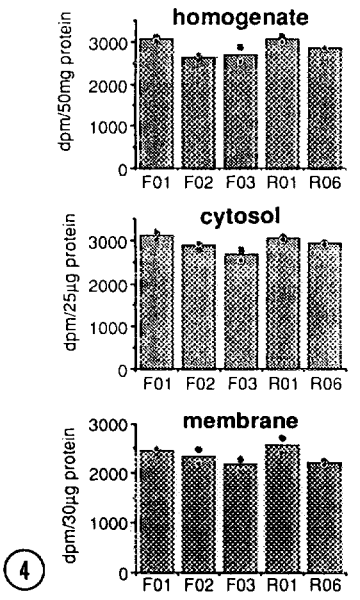


Figure 2. Southern blot analysis of bovine genomic DNA. Restriction fragments were blotted onto a nylon membrane and probed with the radiolabeled full length bovine PLC-α cDNA. The blot was washed and autoradiographed.

Figure 3. Expression of PLC-α in the total cell lysates of NIH3T3 sublines stably transfected with bovine PLC-α cDNA. F01, F02, and F03 are independent NIH3T3 sublines transfected with a sense construct. R01 and R06 are independent cell lines transfected with an antisense construct. Total cell lysates of F01, F02, F03, R01, and R06 cells were immunoblotted with 507 antiserum.

PLC-α is thought to belong to PLC superfamily, but several doubts exist on the authenticity of PLC-α cDNA in view of the following features: (1) no significant homology with any of the known PLCs; (2) relatively lower molecular weight than any of the known PLCs; (3) no evidence that PLC-α cDNA encodes any functional PLC activity [17]. We established NIH3T3 cell lines overexpressing PLC-α protein. Three independent cell lines (F series; F01, F02, and F03) were obtained overexpressing PLC-α protein 20- to 30-fold over the antisense construct-transfected independent cell lines (R series; R01 and R06) (Fig. 3). Lysates were prepared from the homogenates, cytosol, and membrane fractions of these cell lines, and PLC activity was measured with [^{3}H]-labeled phosphatidylinositol 4,5-P_2 as a substrate. We performed the experiments in duplicate and no significant



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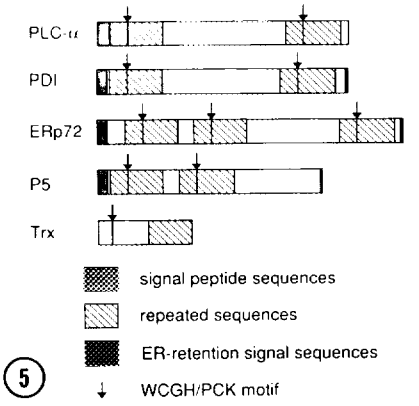

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Figure 4. PLC activities in the homogenates, cytosol, and membrane fractions of the five independent NIH3T3 sublines stably transfected with bovine PLC-α cDNA as described in the legend to Figure 3. Points represent the results of independent experiments with the average of two experiments indicated by bars.

Figure 5. Comparison of protein structures of bovine PLC-α, bovine protein disulfide isomerase (PDI), chinese hamster P5, mouse ERp72, and *E.coli* thioredoxin (Trx). The dotted bars represent the signal peptide; the hatched bars indicate the conserved sequences; the darkened bars indicate ER-retention signals. The Trp-Cys-Gly-His/Pro-Cys-Lys motifs are indicated by arrows.

difference in PLC activity was detected in any of the fractions between F series and R series cell lines (Fig. 4). These data indicate that PLC-α cDNA does not encode any functional PLC activity. PLC-α cDNA was initially cloned from guinea pig uterus by expression cloning with polyclonal antibody raised against the purified protein which had PLC activity [1]. Thereafter, several other groups reported the purification and cDNA cloning of PLC-α as a PLC isotype with PLC activity [18,19]. However, neither group has proven that recombinant PLC-α protein has PLC activity. On the other hand, Martin *et al.* reported an ER-resided protein which is highly homologous to PLC-α but has no PLC activity [20]. Taken together with our result, it is probable that PLC activity was due to the co-purified protein or the degradation of other PLC isoforms and not due to PLC-α itself.

PLC-α contains two stretches of characteristic 110 amino acids within the protein sequence. In each stretch there exists a Trp-Cys-Gly-His-Cys-Lys motif, which is identical to the active sites of protein disulfide isomerase, P5, and ERp72 and highly similar to those of thioredoxin (Fig. 5). Recently, a 60-kDa protein homologous to PLC-α was purified from the rough ER of rat liver and it exhibited thiol-group-related proteolytic activity [21]. Very recently, it was reported that an extract of *E.coli* expressing PLC-α protein showed protein disulfide isomerase activity [22]. Furthermore, we have unraveled that recombinant PLC-α protein contain not PLC activity but thiol-dependent reductase activity (manuscript in preparation). These results indicate that PLC-α could act as a multi-functional protein in the ER.

In conclusion, PLC-α should be excluded from the PLC superfamily and be classified into the gene family which comprises protein disulfide isomerase, P5, ERp72, thioredoxin, and probably others sharing the Trp-Cys-Gly-His/Pro-Cys-Lys motif. We propose that putative PLC-α should be redesignated ERp57 (ER-resided p57).

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REFERENCES

1. Bennett, C. F., Balcarek, J. M., Varrichio, A., and Crooke, S. T. (1988) Nature (London) 334, 268-270.

2. Katan, M., Kritz, R. W., Totty, N., Philip, R., Meldrum, E., Aldape, R. A., Knopf, J. L., and Parker, P. J. (1988) Cell 54, 171-177.

3. Ohta, S., Matsui, Y., Nazawa, Y., and Kagawa, Y. (1988) FEBS Lett. 242, 31-35.

4. Stahl, M. L., Ferenz, C. R., Kelleher, K. L., Kriz, R. W., and Knopf, J. L. (1988) Nature (London) 332, 269-272.

5. Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W., and Rhee, S. G. (1988) Cell 54, 161-169.

6. Emori, Y., Homma, Y., Scrimacki, H., Kawasaki, H., Nakanishi, O., Suzuki, K., and Takenawa, T. (1989) J. Biol. Chem. 264, 21885-21890.

7. Kritz, R., Lin, L. L., Sultzman, L., Ellis, C., Heldin, C. H., Pawson, T., and Knopf, J. L. (1990) CIBA Found. Symp. 150, 112-127.

8. Freedman, R. B. (1989) Cell 57, 1069-1072.

9. Chaudhuri, M. M., Tonin, P. N., Lewis, W. H., and Srinivasan, P. R. (1992) Biochem. J. 281, 645-650.

10. Mazzarella, R. A., Srinivasan, M., Haugejörden, S. M., and Green, M. (1990) J. Biol. Chem. 265, 1094-1101.

11. Holmgren, A. (1989) J. Biol. Chem. 264, 13963-13966.

12. Kato, H., Fukami, K., Shibasaki, F., Homma, Y., and Takenawa, T. (1991) J. Biol. Chem. 267, 6483-6487.

13. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Science 252, 668-674.

14. Nada, S., Okada, M., MacAuley, A., Cooper, J., and Nakagawa, H. (1991) Nature (London) 351, 69-72.

15. von Heijne, G. (1986) Nucleic Acids Res. 11, 4683-4690.

16. Hempel, W. M., and DeFranco, A. L. (1991) J. Immunol. 146, 3713-3720.

17. Meldrum, E., Parker, P. J., and Carozzi, A. (1991) Biochim. Biophs. Acta. 1092, 49-71.

18. Tompkins, T. A., and Moscarello, M. A. (1991) J. Biol. Chem. 266, 4228-4236.

19. Griendling, K. K., Taubman, M. B., Akers, M., Mendlowitz, M., and Alexander, R. W. (1991) J. Biol. Chem. 266, 15498-15504.

20. Martin, J. L., Pumford, N. R., LaRosa, A. C., Martin, B. M., Gonzaga, H. M. S., Beaven, M. A., and Pohl, R. P. (1991) Biochem. Biophys. Res. Commun. 178, 679-685.

21. Urade, R., Nasu, M., Moriyama, T., Wada, K., and Kito, M. (1992) J. Biol. Chem. 267, 15152-15159.

22. Srivastava, S. P., Fuchs, J. A., and Holtzman, J. L. (1993) Biochem. Biophys. Res. Commun. 193, 971-978.