PKC\(\text{II}\), a New Isoform of Protein Kinase C Specifically Expressed in the Seminiferous Tubules of Mouse Testis*

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Yuko S. Niino‡‡, Tarou Irie‡, Mikiro Takaiishi, Tomohiko Hosono‡‡, Nam-ho Huh,†
Tetsuhiko Tachikawa‡, and Toshio Kuroki‡‡

From the ‡Institute of Molecular Oncology, ⁄Department of Pathology, Faculty of Dentistry and ⁄⁄Center for Biotechnology, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555 and the ⁄Department of Cell Biology, Okayama University, Graduate School of Medicine and Dentistry, 2-5-1 Shikatachou, Okayama-shi, Okayama 700-8558, Japan

Protein kinase C (PKC) \(\theta\), a Ca\(^{2+}\)-independent isoform of PKC, has been known to be expressed in skeletal muscle and T cells. In the present study, we isolated and characterized a smaller transcript expressed in the mouse testis, the cDNA of which is referred hereafter as PKC\(\text{II}\) and the original PKC\(\theta\) as PKC\(\theta\). The cDNA clone of PKC\(\text{II}\) has 2184 base pairs and 464 amino acids in the possible open reading frame, consisting of the 5′ unique sequence of 20 amino acids and the PKC\(\theta\) sequence of 444 amino acids. Genomic DNA analysis revealed that transcription of PKC\(\text{II}\) is initiated from the PKC\(\theta\)-specific exon, which is located between exons 7 and 8 of the PKC\(\theta\) gene, indicating that alternative splicing is the mechanism by which PKC\(\text{II}\) is generated. PKC\(\text{II}\) is expressed exclusively in the testis in an age-dependent manner with sexual maturation. In situ hybridization and reverse transcription-polymerase chain reaction of microdissected tissues clearly demonstrated that PKC\(\text{II}\) is expressed in the seminiferous tubules of the mouse testis. Consistent with its molecular structure lacking the C1 regulatory domain, PKC\(\text{II}\) is constitutively active as determined by an in vitro kinase assay, being independent of PKC activators, e.g. phosphatidyserine and phorbol ester. PKC\(\text{II}\) may play a crucial role in spermatogenesis or some related function of the testis.

Protein kinase C (PKC)\(^1\) is a family of serine/threonine kinases that plays crucial roles in signal transduction (1). Members of the PKC family are divided into three groups based on their molecular structures and activating mechanisms: conventional PKC (\(\alpha, \beta, \text{and } \gamma\) isoforms) requiring calcium, phosphatidyserine (PS), and diacylglycerol (DG) for activation; novel PKC (\(\delta, \varepsilon, \eta, \text{and } \theta\) isoforms) activated independent of calcium; and atypical PKC (\(\zeta\) and \(\lambda\)) isoforms), which are independent of both calcium and DG. Alternative splicing-derived variants of PKC were reported for the \(\beta\) and \(\delta\) isoforms, i.e. PKC\(\beta\)I, PKC\(\beta\)II, PKC\(\delta\)I, and PKC\(\delta\)II (2–4).

Among these isoforms, we isolated PKC\(\eta\) and PKC\(\theta\) from a cDNA library of mouse skin (5, 6). Our series of studies elucidated that PKC\(\eta\) is expressed in squamous epithelia in a close association with differentiation (7) and it induces terminal differentiation in keratinocytes by inducing transglutaminase 1 and binding to the cyclin Ecdk2p21 complex (8, 9).

Unlike PKC\(\eta\), PKC\(\theta\) was found to be expressed in muscle cells, suggesting the possibility that it is derived from the muscle layer incidentally present in the skin preparation. Besides muscle cells, PKC\(\theta\) is known to play an important role in T cells; it is colocalized in the T cell receptor and CD3 complex at the contact region between antigen-specific T cells and antigen-presenting cells (10, 11). In T cells, PKC\(\theta\), in synergy with calcineurin, activates JNK and the interleukin-2 promoter and induces cytokine-response modifier A-sensitive apoptosis (12–15). Tang et al. (16) reported that PKC\(\theta\) is also required for angiogenesis and wound healing. Cellular functions mediated by PKC\(\theta\) were reviewed by Meller et al. (17).

Expression of a small-size PKC\(\theta\) mRNA in the testis was reported by Mischak et al. (18). We also noted the presence of a small-size transcript of PKC\(\theta\) in the testis.\(^2\) However, this small-sized PKC\(\theta\) has not been characterized yet.

In the present study, we report on PKC\(\text{II}\), a new PKC isoform derived by alternative splicing of the PKC\(\theta\) gene, which lacks the V1 domain and a zing finger motif in the C1 regulatory domain and is expressed exclusively in the seminiferous tubules of the mouse testis.

**EXPERIMENTAL PROCEDURES**

Library Screening and DNA Sequencing—The mouse testis cDNA library prepared from the CD-1 mouse testis (Stratagene, CA) was screened by a standard protocol (19) using PCR products of the V3 region (875–1289 bp) of PKC\(\theta\) as a probe (probe A in Fig. 3A). A pair of the primers 1/2 used is shown below in Table I. Among the \(\sim 1 \times 10^6\) independent colonies screened, two clones were isolated, which were then subjected to DNA sequencing with the ALFexpress system (Amersham Pharmacia Biotech, UK).

5′-Rapid Amplification of cDNA Ends—The 5′-end of PKC\(\text{II}\) mRNA was determined using the 5′RACE kit (version 2, Life Technologies, Inc., Gaithersburg, MD) according to the conditions suggested by the manufacturer. PCR products were subcloned into \(\phi\)BluescriptII(−) and sequenced with the ALFexpress system.

Northern Blot Analysis—The Mouse Multiple Tissue Northern Blot Analysis (Clontech) was performed according to the manufacturer’s protocol. The probe used was a 165-bp fragment of PKC\(\text{II}\) mRNA, derived from cDNA of PKC\(\text{II}\) clone by RT-PCR, which contains 145-nucleotide sequence specific for PKC\(\text{II}\).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^7\)/EBI Data Bank with accession number(s) AB062122.

‡ To whom correspondence should be addressed: Dept. of Anatomy, Showa University, School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Tel.: 81-3-3784-8104; Fax: 81-3-3784-8115; E-mail: niino@med.showa-u.ac.jp.

† The abbreviations used are: PKC, protein kinase C; PS, phosphatidyserine; TPA, 12-0-tetradecanoylphorbol-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DG, diacylglycerol; JNK, c-Jun N-terminal kinase; bp, base pairs; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; CMV, cytomegalovirus; PMSF, phenylmethylsulfonyl fluoride; crmA, cytokine-response modifier A.

\(^2\) Y. S. Niino, T. Irie, M. Takaishi, T. Hosono, N.-h. Huh, T. Tachikawa, and T. Kuroki, unpublished data.

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(MTN) Blot (CLONTECH, Palo Alto, CA) was hybridized with random-prime-labeled cDNA probes: these were probe A, mentioned above for PKCθII, 50–250 bp of PKCθI (probe B in Fig. 3A) for PKCθII, and 118–577 bp of PKCθII (probe C in Fig. 3A) for PKCθII. The filters were exposed overnight for 3 nights (PKCθI and PKCθII) or for 7 nights (PKCθII) consecutively. 

**Genomic PCR**—Genomic DNA of an 8-week-old female C57BL/6 mouse was prepared from the tail by a method described previously (20) and subjected to genomic PCR using the Expand Long Template PCR system (Roche Molecular Biochemicals, Germany) under conditions recommended by the manufacturer. Pairs of the primers 3/5, 4/5, and 6/7 were used for amplification of exon 2/θ exon, exon 4/θ exon, and θ II exon/exon 8, respectively (Table I). PCR products were subcloned into plBluescriptII (+) and sequenced by Nippon Flour Mills Co., Ltd.

**RT-PCR of Testis**—Total RNA of the testis from 0-, 1-, 2-, 3-, 4-, 8- and 26-week-old mice was purified with a QuickPrep Total RNA extraction kit (Amersham Pharmacia Biotech, UK). Five micrograms of total RNA was reverse-transcribed in the reaction mixture containing 50 mM Tris-acetate, pH 8.4, 75 mM potassium acetate, 8 mM magnesium acetate, 0.01 M dithiothreitol, 2 mM dNTP, 25 μM oligo(dT)12-18, 25 μg/mL random hexamer oligonucleotides, and avian RNase H-minus reverse transcriptase (Life Technologies, Inc.) for 60 min at 50 °C. The resulting cDNAs were amplified in 25 μL of PCR mix consisting of GeneAmp 1× PCR Gold Buffer (PerkinElmer Life Sciences), 1.5 μg MgCl2, 200 μM dNTP, and 0.125 unit of AmpliTaq Gold (PerkinElmer Life Sciences) under the following conditions: 10 min at 95 °C, 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by 7 min at 72 °C. Pairs of the primers used were primers 8/10 for PKCθII, 9/10 for PKCθII, 15/16 for β-actin, 17/18 for proteamine-I, and 19/20 for selenoprotein-P. 

**FLAG-tagged Expression Plasmids**—FLAG-tagged expression plasmids of PKCθI and θII were constructed with pFLAG-CMV5 (Eastman Kodak, Rochester, NY). The stop codon was removed from cDNAs of PKCθI and PKCθII, and the EcoRV site was introduced by PCR. Both cDNAs were subcloned into the EcoRV site of pFLAG-CMV5. The cDNAs were transfected using the LipofectAMINE 2000 reagent (Life Technologies, Inc.) into HEK293 cells grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. The cells were harvested 24 h after transfection for immunoprecipitation.

**Immunoprecipitation**—Full-length cDNAs of PKCθI and θII were cloned into pEF-BOS expression plasmid (23) and transfected into COS7 cells, which were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum for 24 h. Transfected COS7 cells as well as testis from 26-week-old mouse (50 mg each) were sonicated in homogenizing buffer, and aliquots of each samples were subjected to immunoblotting with anti-PKCθ antibody against the C-terminal peptide for PKCDI/II (nPKCθ (C-18), Santa Cruz Biotechnology, Santa Cruz, CA). Transfected HEK293 cells were sonicated in a homogenizing buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl2, 5 mM EGTA, 1% Nonidet P-40, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL antipain, and 5 μg/mL aprotinin). The supernatants obtained by centrifugation at 21,000 × g for 10 min were incubated with Protein G-Sepharose beads harboring anti-FLAG antibody (Sigma Chemical Co., St. Louis, MO), followed by washing with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL antipain, and 5 μg/mL aprotinin) three times, buffer B (the same as buffer A without EGTA) once, and buffer C (20 mM Tris-HCl, pH 7.5, 50 mM MgCl2, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL antipain, and 5 μg/mL aprotinin) once. An aliquot of the immunoprecipitated beads was separated using 8% SDS-polyacrylamide gel electrophoresis and transferred onto a Hybond-C membrane (Amersham Pharmacia Biotech, UK). Transfected products on membranes were blocked with 5% skim milk and 3% bovine serum albumin in phosphate-buffered saline and incubated with the anti-PKCθ antibody, followed by immunodecoration using Western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Expression at the protein level was monitored using a chemiluminescence detection system (Amersham Life Sciences).

**List of primers used for PCR**

| Primer no. | Gene     | Region     | Direction | Primer                                    |
|-----------|----------|------------|-----------|-------------------------------------------|
| 1         | PKCθII   | V3         | Sense     | 5′-CTGAAGCAGCTGCGATGATTG                  |
| 2         | PKCθII   | V3         | Antisense | 5′-GCTAGATCTTCCGCTTCCACG                 |
| 3         | PKCθII   | θII exon   | Sense     | 5′-CTCTGGGACCAGAAATATTG                  |
| 4         | PKCθII   | exon 4     | Sense     | 5′-GTCTCCCATGATTTCCTG                   |
| 5         | PKCθII   | θII exon   | Antisense | 5′-GACTATTTGCTCCCTCCAGAC                 |
| 6         | PKCθII   | exon 8     | Sense     | 5′-ATCGTTGCGAGATTTCCCG                  |
| 7         | PKCθII   | C1         | Antisense | 5′-TCATAAGCGGAGAAATATTG                 |
| 8         | PKCθII   | θII exon   | Sense     | 5′-GGTATCTTCTCCTGTGCGTTCGTA            |
| 9         | PKCθII   | V1         | Antisense | 5′-CAATGGCAGCAAATATTG                   |
| 10        | PKCθII   | C1         | Antisense | 5′-GCAAGCTTTTGATGGAG                     |
| 11        | PKCθII   | θII exon   | Antisense | 5′-ATCTCTGTCAATGAGGTCA                   |
| 12        | PKCθII   | θII exon   | Antisense | 5′-GTTGGACTGTTAGG                        |
| 13        | PKCθII   | θII exon   | Antisense | 5′-CAATGACGCGTACAGG                      |
| 14        | PKCθII   | θII exon   | Antisense | 5′-CAATGACGCGTACAGG                      |
| 15        | β-Actin  |            | Sense     | 5′-GACCTCTTACGGCAACACACAGTG             |
| 16        | β-Actin  |            | Antisense | 5′-TGCTGATCCACATCTCGCTGGA            |
| 17        | Proteamine-I |        | Antisense | 5′-AGGGTGGCTGCTGCGGCCG                  |
| 18        | Proteamine-I |        | Antisense | 5′-GGTCATCTTCTCGTGTGCCG                  |
| 19        | Selenoprotein-P | | Antisense | 5′-CTATCAGGTTGAAACTGAC                   |
| 20        | Selenoprotein-P | | Antisense | 5′-GACCTCTTACGGCAACACACAGTG             |
| 21        | G3PDH    |            | Sense     | 5′-CTATCAGGTTGAAACTGAC                   |
| 22        | G3PDH    |            | Antisense | 5′-CAATGACGCGTACAGG                      |
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In Vitro Kinase Assay—Kinase activity of pFLAG-PKCαII and -PKCαII was assayed in vitro using myelin basic protein (Sigma) as a substrate. Immunoprecipitated proteins were normalized by immunoblotting and incubated in 50 μl of the reaction mixture (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 100 μM ATP, 1 μM Pb2+, 5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin) in the presence or absence of the PKC activators, e.g. 1 mM CaCl2 or 1 mM EGTA, 50 μg/ml PS, and 100 nm PMA at 25 °C for 10 min. The reaction was stopped by adding 30 μl of SDS sample buffer and boiling for 3 min. Following a brief centrifugation, 20-μl aliquots of supernatants were subjected to 15% SDS-polyacrylamide gel electrophoresis, and after drying, the gels were exposed to an x-ray film.

RESULTS

Isolation of PKCαII cDNA—As shown in Fig. 1, Northern blot analysis of PKCα in mouse tissues demonstrated the existence of at least four transcripts corresponding to bands of 3.5-, 3.0-, 2.5-, and 1.2-kb mRNA when a DNA fragment of the V3 region was used as a probe (probe A in Fig. 3A). The two large transcripts (3.5 and 3.0 kb) were detected in all the tissues examined except for the testis, whereas the testis expressed smaller transcripts of 2.5 and 1.2 kb.

To identify the transcript of 2.5 kb, we screened a mouse testis cDNA library with probe A and isolated two independent clones containing the entire coding region. Throughout this paper, the 2.5-kb transcript is referred to as PKCαII and the original PKCα as PKCαI. The transcript of 1.2 kb remains to be identified.

The cDNA clone of PKCαII has 2184 bp, to which 18 nucleotides were added by 5′-RACE of mouse testis mRNA. As shown in Fig. 2, PKCαII cDNA consists of a unique sequence of 276 bp at the 5′-end and a sequence identical to PKCαI at the 3′-end, of which the boundary is located at 879 bp of the PKCαI cDNA (GenBank® accession number D11091). The deduced amino acid sequence of PKCαII contains four methionines, as a candidate of the starting amino acid for translation, although none of them conform to the Kozak consensus sequence (Fig. 2). Hereafter, we adopted the first methionine as a starting site because of the presence of an in-frame termination codon upstream.

A possible open reading frame initiated from the first ATG codes 464 amino acid, consisting of the 5′ unique sequence of 20 amino acids and the PKCαI sequence of 444 amino acids (Fig. 2). The latter sequence contains a part of the C1 regulatory domain lacking the zinc finger motif, the V3 domain, the C3/V4/C4 catalytic domain, and the C-terminal V5 domain. The N-terminal 20-amino acid sequence does not show significant homology to any sequence in the existing cDNA database. The structure of the PKCαII cDNA is compared with that of PKCαI in Fig. 3A.

Testis-specific Transcription of PKCαII—Northern blot analysis showed that the unique sequence of PKCαII (probe B in Fig. 3A) hybridized only the smaller PKCαII transcripts expressed in the testis (Fig. 1B), whereas a probe of the 5′-end of PKCαI (probe C in Fig. 3A) hybridized mRNA of PKCαI expressed in the heart, brain, spleen, lung, liver, skeletal muscle, and kidney (Fig. 1C). A common probe for PKCαI and PKCαII (probe A) hybridized both PKCαI and PKCαII (Fig. 1A). In addition, we found that PKCαII was not expressed in the ovary (data not shown). These results indicate that PKCαII is specifically transcribed in the testis whereas PKCαI is expressed ubiquitously except in the testis.

Genomic Structure of PKCα Gene—The above sequencing data suggest that the 2.5-kb transcript detected in the testis could be derived from alternative splicing of the PKCα gene (Pckα). The Pckα was mapped to chromosome 10p15 in human (24) and chromosome 2 in mice (25). Its genomic structure in human was reported (GenBank® accession number AL158043).

PCR analysis of the mouse genomic DNA indicated that the sizes of PCR products from the PKCαII-specific domain to exons 2, 4, and 8 were 20, 5.5, and 2.3 kb, respectively. Exons 4, 5, 6, 7, and 8 were found to be well conserved in mouse and human genomes. Based on these results, we located the PKCαII-specific exon composed of 276 nucleotides (hereafter, the αII exon) between exons
Age-dependent expression of PKC\(_{\text{II}}\) in mouse testis. Total RNA isolated from 0-, 1-, 2-, 3-, 4-, 8-, and 26-week-old mice testis was subjected to RT-PCR, using primers specific to PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\).

Fig. 5A, total proteins of the testis from 26-week-old mouse as well as from COS7 cells transfected with PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\) cDNAs were subjected to immunoblotting with the antibody against to the common C-terminal peptide for PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\). A band corresponding to the molecular mass of PKC\(_{\text{II}}\) (50 kDa) was detected in the testis and PKC\(_{\text{II}}\)-transfected cells, whereas a band corresponding to PKC\(_{\text{I}}\) (80 kDa) was seen only in the PKC\(_{\text{I}}\)-expressing cells. Expression at the protein level was further confirmed by immunoprecipitation-immunoblotting of HEK293 cells transfected with pFLAG-PKC\(_{\text{I}}\) and -PKC\(_{\text{II}}\) (Fig. 5B). The transfected HEK293 cells were immunoprecipitated with the anti-FLAG antibody, followed by immunoblotting with the PKC\(_{\text{I}}\)/PKC\(_{\text{II}}\) antibody. Again, products of PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\) were detected in the cells transfected with the corresponding cDNAs.

Localization of PKC\(_{\text{II}}\) in Testis—The testis is composed of

7 and 8 (Fig. 3B). Exon \(\theta\)I can be accepted into exon 8, because sequences at the exon-intron boundaries fit with the donor-acceptor splicing rule. However, no splicing acceptor sequence was found around the 5'-boundary of the \(\theta\)I exon, suggesting that the \(\theta\)I exon is the first exon of PKC\(_{\text{II}}\) cDNA.

Fig. 3B summarizes the generation of PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\) cDNAs from the PKC\(_{\text{I}}\) gene by alternative usage of exons: The V1 and C1 domains of PKC\(_{\text{I}}\) cDNA are generated from exons 1–8, whereas the PKC\(_{\text{II}}\) unique sequence is derived from the \(\theta\)I exon. A testis-specific promoter may be located upstream of the \(\theta\)I exon. This genomic structure indicates that alternative splicing is the mechanism by which PKC\(_{\text{II}}\) is generated.

Age-dependent Expression of PKC\(_{\text{II}}\)—In the testis, spermatogenesis and androgen production proceed in an age-dependent manner, becoming mature at about 4 weeks after birth. Age-dependent expression of PKC\(_{\text{II}}\) was analyzed by RT-PCR using total testis RNA isolated from newborn, 1-, 2-, 3-, 4-, 8-, and 26-week-old mice. Expression levels were normalized to GAPDH, and developmental stages were monitored based on the expression of c-Kit, protamine-1, and acrosin (data not shown).

When the primers for PKC\(_{\text{II}}\)-specific sequences were used, expression of PKC\(_{\text{II}}\) was first detected at 3 weeks and increased thereafter with age until adulthood (Fig. 4). In contrast, PKC\(_{\text{I}}\) was not expressed in the testis at any age when amplified using the PKC\(_{\text{I}}\)-specific primers.

This observation suggests that PKC\(_{\text{II}}\) is involved in the maturation of a cell function(s) in the testis, e.g., spermatogenesis and/or androgen production, and responsiveness.

Expression of PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\) at the Protein Level—PKC\(_{\text{I}}\) and PKC\(_{\text{II}}\) were expressed at the protein level in the mouse testis and in the cells overexpressing these cDNAs. In
two important functional components: the seminiferous tubules producing male germ cells and the interstitium producing androgen. To localize the expression of PKC\(\alpha\)II in the testis, we performed in situ hybridization and RT-PCR of laser-microdissected tissues. As shown in Fig. 6, mRNA transcribed by the \(\alpha\)II exon was detected by in situ hybridization exclusively in the seminiferous tubules, not in the interstitial cells. A negative control with a sense probe did not show any signal. Furthermore, PKC\(\alpha\)II was found not to be expressed in the testis when the PKC\(\alpha\)II-specific sequence was used as a probe (data not shown). It is not certain from in situ hybridization whether Sertoli cells expressed PKC\(\alpha\)II, although a Sertoli cell-derived cell line, TM-4, did not express it (data not shown).

These specific features of PKC\(\alpha\)II expression were further confirmed by RT-PCR of the microdissected seminiferous tubules and interstitial cells from 2- and 4-week-old mice (Fig. 7). The expression of PKC\(\alpha\)II was monitored based on \(\beta\)-actin mRNA, and cell type specificities were examined morphologically as well as expression of protamine-1 and selenoprotein-1 in the seminiferous tubules and interstitium, respectively (26, 27). As shown in Fig. 7B, PKC\(\alpha\)II was expressed in the seminiferous tubules of 4-week-old mice, but not in those of 2-week-old mice, whereas no expression was observed in the interstitium. PKC\(\alpha\)II was not detected in any testis component at 2 or 4 weeks.

These data clearly indicate the exclusive expression of PKC\(\alpha\)II in seminiferous tubules in an age-dependent manner.

Kinase Activity of PKC\(\alpha\)II and PKC\(\alpha\)II—The lack of the complete C1 regulatory domain in PKC\(\alpha\)II suggests the possible independence of PS and DG or phorbol esters for its activation. To address this question, cDNAs of PKC\(\alpha\)I and PKC\(\alpha\)II were subcloned into the expression vector pFLAG-CMV5, transfected into HEK293 cells, and immunoprecipitated with the anti-FLAG antibody. After normalizing the expression level of PKC\(\alpha\)I and PKC\(\alpha\)II by immunoblotting with the anti PKC\(\alpha\) antibody (Fig. 5B), immunoprecipitates were subjected to in vitro kinase assay in the presence or absence of known activators of PKC. Myelin basic protein was used as a substrate. As seen in Fig. 5A, PKC\(\alpha\)II showed a certain level of kinase activity in the absence of activators and no further activation was observed following the addition of Ca\(^{2+}\), PS, or TPA, suggesting the possibility that PKC\(\alpha\)II is constitutively active independent of the PKC activators. Activity of PKC\(\alpha\)I, however, was dependent on PS and TPA, which is in agreement with previous report (6, 28).

In contrast to phosphorylation of myelin basic protein, autophosphorylation was observed only with PKC\(\alpha\)I in an activator-dependent manner (Fig. 8B). No autophosphorylation was noted with PKC\(\alpha\)II.

Discussion
PKC\(\alpha\)II is unique in that it is mainly composed of a catalytic domain and it is specifically expressed in the seminiferous tubules of the mouse testis. Genomic DNA analysis revealed that PKC\(\alpha\)II is generated by alternative splicing of the PKC\(\alpha\) gene. The cDNA of PKC\(\alpha\)II consists of a unique N-terminal sequence encoding 20 amino acids and the C-terminal sequence identical to that of PKC\(\alpha\)I encoding a part of the C1 regulatory domain followed by the V3 domain, C3/V4/C4 catalytic domain, and V5 domain.

Alternative splicing is a common transcriptional mechanism, by which functionally diverse polypeptides are produced from a single gene. Based on a minimum estimate, 35% of human genes show variably spliced products (29, 30). Alternative splicing yields a wide variety of the encoded proteins by addition or deletion of a sequence(s), which may be involved in a certain stage of development and differentiation.
PKC0II, a New Isoform of Protein Kinase C

FIG. 7. Exclusive expression of PKC0II in the microdissected seminiferous tubules. A, seminiferous tubules (a–c) and interstitium (d–f) of mouse testis at 2 and 4 weeks old were microdissected. Specimens before (a, d) and after (b, e) microdissection, and after laser pressure cell transfer (c, f) are shown. B, RT-PCR of the microdissected tissues from 2-week-old (top) and 4-week-old (bottom) mice using PKC0I- and PKC0II-specific primers. Tissue specificities were monitored based on morphology (A) and expression of marker genes (p, protamine-1 for seminiferous tubules; s, selenoprotein-P for interstitium; a, β-actin for amount of RNA).

FIG. 8. Kinase activity of PKC0I and PKC0II in terms of phosphorylation of myelin basic protein (A) and autophosphorylation (B). pFLAG-CMV, pFLAG-PKC0I, and pFLAG-PKC0II were transfected into HEK293 cells, immunoprecipitated with the anti-FLAG antibody, and subjected to in vitro kinase assay. Phosphorylation was detected in the presence or absence of known activators of PKC: Lane 1, no activators; lane 2, 1 mM CaCl2; lane 3, 50 μg/ml PS; lane 4, 100 ng/ml TPA and lane 5, 50 μg/ml PS and 100 ng/ml TPA.

As for PKC, alternatively spliced isoforms of the β and δ isoforms are known. PKC0βI and -δI are produced by alternative splicing of the C-terminal V5 domain, which may function in intracellular localization (31, 32). Recently, two alternatively spliced isoforms of PKCδ have been reported, i.e. PKCδII and PKCδIII. PKCδII contains a 78-bp insertion at the caspase-3 recognition sequence at the V3 domain (GenBank® accession number AB011812). PKCδIII is generated by an 83-bp insertion at the same V3 domain, causing in-frame termination, thereby yielding a truncated form of PKCδ without the catalytic domain (4). In the present study, we reported another alternatively spliced isoform of PKC, i.e. PKC0II.

Functions of certain proteins are switched on/off by phosphorylation and dephosphorylation. Switching enzymes, therefore, should be strictly regulated. There are several ways of regulating protein kinases. These include binding of the regulatory proteins, a good example being cdk kinases that are regulated by binding to cyclins and cdk inhibitors, and phosphorylation of a specific residue, such as serine 15 of p53 and threonine 160 of cdk2 (33, 34). PKC is well known to be activated by metabolic turnover of polar head groups of membrane phospholipids (35). Other activation mechanisms for PKC include phosphorylation of a serine/threonine residue at the activation loop (36) and cleavage between the regulatory and catalytic domains (37). The latter mechanism is the case for PKC0II.

When assayed in vitro using immunoprecipitated PKC0II, a certain level of background kinase activity was noted in the absence of PS and TPA. Addition of activators did not enhance the activity, which is consistent with its molecular structure, i.e. lacking the zinc finger motif in the C1 regulatory domain. These data suggest that PKC0II is constitutively active independent of PKC activators. PKC0II may be regulated at the transcription level or by a yet-unidentified mechanism. Absence of autophosphorylation in PKC0II suggests possible absence of an autophosphorylation site(s) or its regulating mechanisms in PKC0II.

PKC0II is most unique in that it is expressed exclusively in germ cells in seminiferous tubules. There is no expression of PKC0II in any tissue other than the testis, and in the testis the expression is limited exclusively to seminiferous tubules. This was demonstrated by in situ hybridization and RT-PCR of the microdissected tissues using the PKC0II-specific probe or primer, respectively. In contrast, Kim and Shin (38) reported that signals of PKC0 were detected in the interstitial cells of mouse testis by in situ hybridization and immunohistochemical staining. However, the 5'-end probe that they used for in situ hybridization was specific to PKC0I, not to PKC0II, and the commercially available antibody used recognized both PKC0I and PKC0II. We also found that the same antibody stained interstitial cells not seminiferous tubules.

Spermatogenesis occurs in seminiferous tubules and consists of three phases: proliferation and differentiation of spermatocytes...
gonia, meiotic division of spermocytes, and development of post-haploid spermatids to sperms. Age-dependent expression of PKCθII after 3 weeks coincides with differentiation of haploid germ cells, suggesting its crucial involvement in spermatogenesis. However, Sun et al. (39) reported that PKCθ null mice seemed normal and were fertile. These mice were generated by homologous deletion of the exon encoding the ATP binding site of the C3 domain, which corresponds to amino acid residues 154–207 in PKCθII. Fertility of these mice may be due to redundancy of PKC in testis, in which α, δ, θII, and γ isoforms are present (18). Further study is needed for elucidating the mechanism by which PKCθII mediates signals for spermatogenesis.

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