RESEARCH COMMUNICATION

Cloning and mapping of human *PKIB* and *PKIG*, and comparison of tissue expression patterns of three members of the protein kinase inhibitor family, including PKIA

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Two novel members of the human cAMP-dependent protein kinase inhibitor (PKI) gene family, *PKIB* and *PKIG*, were cloned. The deduced proteins showed 70 % and 90 % identity with mouse PKIβ and PKIγ respectively. Both the already identified pseudosubstrate site and leucine-rich nuclear export signal motifs were defined from the 11 PKIs of different species. The *PKIB* and *PKIG* genes were mapped respectively to chromosome 6q21-22.1, using a radiation hybrid GB4 panel, and to chromosome 20q13.12-13.13, using a Stanford G3 panel. Northern-blot analysis of three PKI isoforms, including the PKIA identified previously, revealed significant differences in their expression patterns. *PKIB* had two transcripts of 1.9 kb and 1.4 kb. The former transcript was abundant in both placenta and brain and the latter was expressed most abundantly in placenta, highly in brain, heart, liver, pancreas, moderately in kidney, skeletal muscle and colon, and very little in the other eight tissues tested. *PKIG* was widely expressed as a 1.5-kb transcript with the highest level in heart, hardly detectable in thymus and peripheral blood leucocytes and was moderately expressed in the other tissues, with slightly different levels. However, PKIA was specifically expressed as two transcripts of 3.3 kb and 1.5 kb in heart and skeletal muscle. The distinct expression patterns of the three PKIs suggest that their roles in various tissues are probably different.

Key words: human *PKIB* and *PKIG*, protein kinase inhibitor motif, tissue-expression pattern.

INTRODUCTION

The protein kinase inhibitors (PKIs) are a class of proteins that can inhibit the activity of cAMP-dependent protein kinase (PKA), a complex composed of two regulatory subunits (R-subunits) and two catalytic subunits (C-subunits). The function of PKA mainly depends on its free C-subunits, which leave the inactive tetrametric complex in the cytoplasm in response to an increase in intracellular cAMP concentration [1]. The PKI binds the C-subunits of PKA in the nucleus and carries them into the cytoplasm to reform the inactive PKA complex with R-subunits. Studies on the molecular structure of human and/or mouse PKA, have shown that the R-subunit includes four isoforms (RIα, RIβ, RIIα and RIIβ) and the C-subunit contains three isoforms (Cα, Cβ and Cγ) [2]. To date, three mouse *Pki* genes encoding three isoforms (PKIα, PKIβ and PKIγ) have been cloned [3–5]. Although all three PKI proteins can inhibit PKA activity, they may act by preferentially inhibiting different C-subunits [6]. However, only one human *PKI* gene (termed *PKIA*), which is homologous to mouse *Pki*α, has been cloned and its protein product inhibits the activity of PKA [1]. Thus it is speculated that other PKI members might also exist in humans. Before the inhibition of the various C-subunits by different PKIs in humans can be elucidated, the identification of additional human *PKI* genes is necessary. In the present study, the isolation of two novel human *PKI* genes (*PKIB* and *PKIG*) is described, and a comparison of the expression patterns of the genes in the *PKI* family, including *PKIA*, is made.

EXPERIMENTAL

The cDNA sequences of mouse *Pki*β (GenBank accession number L02241) and *Pki*γ (GenBank accession number U97176) were used to search the Human EST (expressed sequence tag) division of GenBank. Homologous ESTs were obtained and assembled into two EST contigs using Assembly Machine (http://gcg.tigem.it/cgi-bin/uniestass.pl). Two pairs of primers, LZB_1 (5′-GCCAGGGACAGGAAAGATAGGAG-3′) and LZB_2 (5′-CACATGCTGCTTAGGGCTGCAATG-3′); LZG_1 (5′-ACAGGCCTGAGGAGCGATGCAC-3′) and LZG_2 (5′-GGAGCTCAGAGAAGAGGCTGCTG-3′) were designed based on the contig sequences, and custom-made by ShengGong Inc. (Shanghai, China). The primers were used to amplify several human tissue cDNA λgt11 libraries (Clontech, Palo Alto, CA, U.S.A.). PCR conditions with primers LZB_1 and LZB_2, and primers LZG_1 and LZG_2 were as follows: 1 μl template ($> 10^8$ plaque-forming units/ml) was amplified in a final volume of 50 μl containing 5 μl of 10 $\times$ PCR buffer, 1 μl of 20 mM dNTPs, 1.5 μl of 2.5 mM $MgCl_2$, 2 units *Taq* polymerase (Promega) and 1 μl of 25 mM of each specific primer. PCR reactions were run on a PTC-200 DNA Engine (MJ Research, Watertown, MA, U.S.A.) for 34 cycles (1 min at 93 °C, 1 min at 60 °C and 1 min

Abbreviations used: EST, expressed sequence tag; PKA, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; RH, radiation hybrid; UTR, untranslated region.

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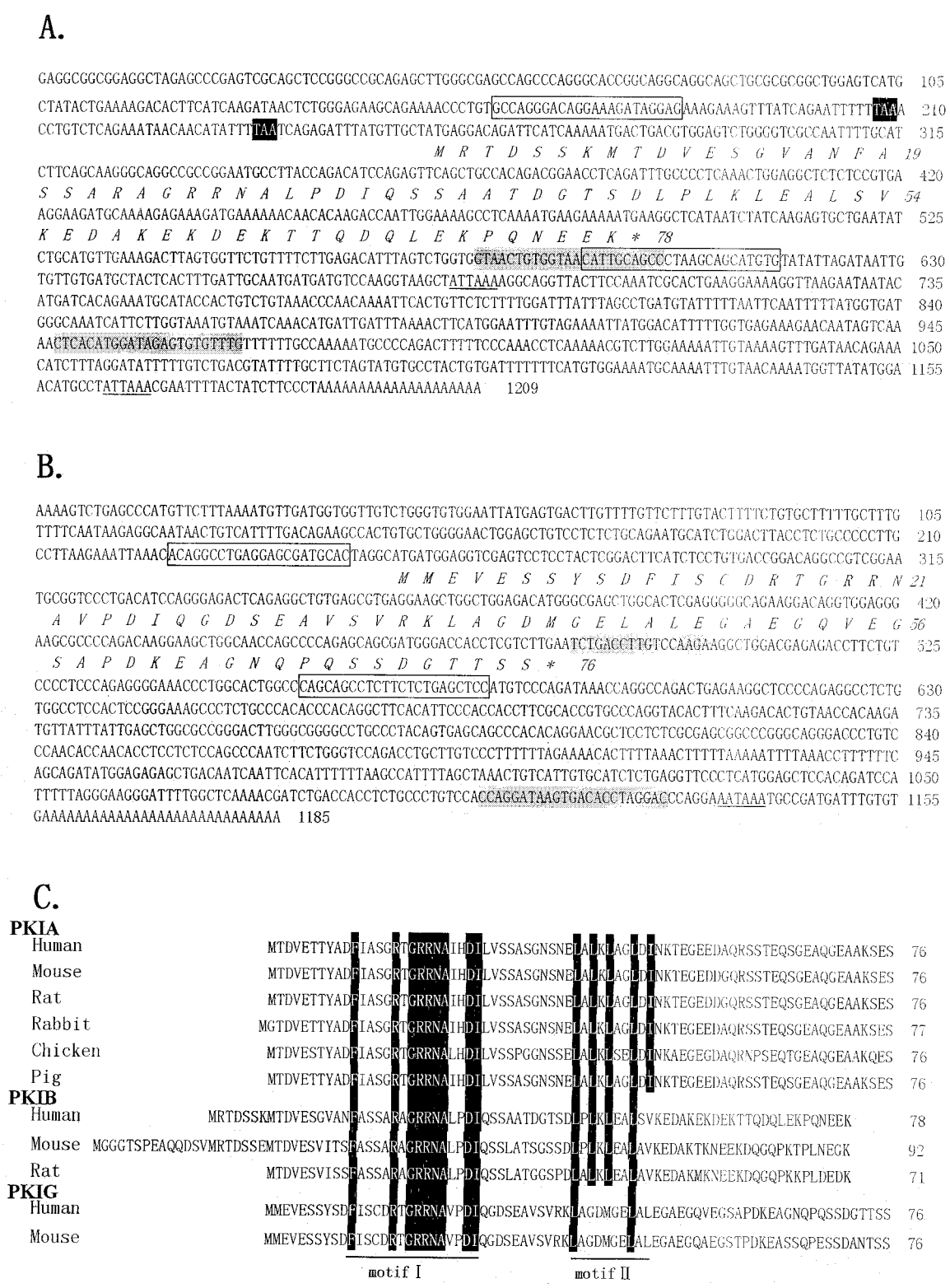

Figure 1 cDNA and deduced amino acid sequences of human *PKIB* (A) and *PKIG* (B), and multiple alignment of PKIs from different species to show PKI motifs (C)

(**A**) and (**B**) Primers for PCR amplification (LZB_1 and LZB_2; LZG_1 and LZG_2) are boxed, and for RH mapping (LZB-RH_1 and LZB-RH_2; LZG-RH_1 and LZG-RH_2) are shown with grey shading. Polyadenylation signal sequences (ATTAAA) are underlined. In-frame stop codons (TAA) in the upstream of the start codon of *PKIB* are shown with a black background. (**C**) PKIs from different species were aligned using the CLUSTAL program. The pseudosubstrate site (motif I) and leucine-rich nuclear export signal (motif II) are shown with a black background. The GenBank accession numbers of the PKI sequences are as follows. PKIA: Human, S76965; Mouse, M63554; Rat, L02615; Rabbit, M23079; Chicken, U19496; Pig, AF132737. PKIB: Human, AF087873; Mouse, L02241; Rat, M64092. PKIG: Human, AF115966; Mouse, U97170.

at 72 °C) after an initial denaturation at 93 °C for 3 min, and were followed by incubation at 72 °C for 5 min. Based on the human *PKIA* sequence reported previously [1], a pair of primers, LZA_1 (CCTGCTATGTGGATATTTGGTAG) and LZA_2 (GGTTCCTGCAATGCAGCACAGCCA) was designed and used to amplify cDNA libraries as described above. The PCR products were subcloned into the pGEM-T vector and sequenced using the BigDye terminator sequencing kit and ABI377 sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.).

The protein sequences of PKI isoforms from other organisms were searched for in Entrez (National Center for Biotechnology Information) and motif analysis was performed using the MEME program (Multiple EM for Motif Elicitation; http://www.sdsc.edu/MEME/meme/website/) [7].

Radiation hybrid (RH) mapping of *PKIB* was performed using a GB4 panel (GeneBridge4 Human/Hamster RH Panel; Research Genetics Inc., Huntsville, AL, U.S.A.) and PCR primers $LZB\text{-}RH_1$ (5′-GTAACTGTGGTAACATTGCAGCC-3′) and $LZB\text{-}RH_2$ (5′-CAAACACACTCTATCCATGTGAG-3′). The primers were designed, based on the 3′-untranslated region (UTR) sequence of *PKIB* cDNA. The PCR conditions were as above and the reactions were run for 40 cycles (1 min at 94 °C, 1 min at 63 °C and 40 s at 72 °C) after initial denaturation at 94 °C for 3 min, and were followed by incubation at 72 °C for 10 min. The PCR products, amplified with DNA samples from five Chinese subjects, were sequenced to confirm that they were identical to the 3′-UTR sequence of *PKIB* cDNA. The PCR results were graded (0 if two duplicate PCRs showed a negative signal, 1 if both showed a positive signal and 2 if they showed a different signal) and were sent to the Sanger RH Mapping Server (http://www.sanger.ac.uk/RHserver/RHserver.shtml) for statistical analysis. RH mapping of *PKIG* was determined using a G3 panel (Stanford G3 Human/Hamster RH Panel; Research Genetics Inc.). The PCR primers used were $LZG\text{-}RH_1$ (5′-TCTGACCTTGTCCAAGAAGGCTG-3′) and $LZG\text{-}RH_2$ (5′-GTCCTAGGTGTCACTTATCCTGG-3′); the annealing temperature for each PCR amplification cycle was 60 °C. The procedure was then the same as for the RH mapping of *PKIB* described above. The recorded PCR results of *PKIG* were sent to the Stanford RH mapping server (http://www-shgc.stanford.edu/RH/rhserverformnew.html) for statistical analysis. The chromosome locus was determined using the GDB database (http://gdbwww.gdb.org/gdb/).

Northern hybridizations of *PKIA*, *PKIB* and *PKIG* were performed on multiple-tissue Northern (MTN) membranes (MTNI and MTNII; Clontech Inc.) with mRNA samples from 16 adult human tissues. The probes were prepared by labelling the cDNA fragments (amplified from human cDNA libraries as described above) with [α-^{32}P]dATP (Amersham) using the PCR method and were purified on a Sepharose-G50 column. The MTN membranes were prehybridized in hybridization/prehybridization solution {5 × SSPE [1 × SSPE = 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 50 % formamide, 10 × Denhart's (1 × Denhardt's = 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.002 % BSA), 2 % (w/v) SDS, 100 μg/ml calf-thymus DNA} at 42 °C for 16 h, and hybridized in a hybridization oven (Hybaid Interactive, Teddington, Middx., U.K.) with the labelled probe for a further 24 h, with continuous shaking. The membranes were then washed several times with 0.1 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1 % SDS at 42 °C and were exposed to X-ray film at −80 °C for 5 days. Northern blotting of human β-actin was performed as described above, except that the membranes were exposed to the X-ray film for 8 h. The intensities of the bands were determined using the Complete Gel Documentation and Analysis System (GDS 8000; Gene Company Limited, Shanghai, China).

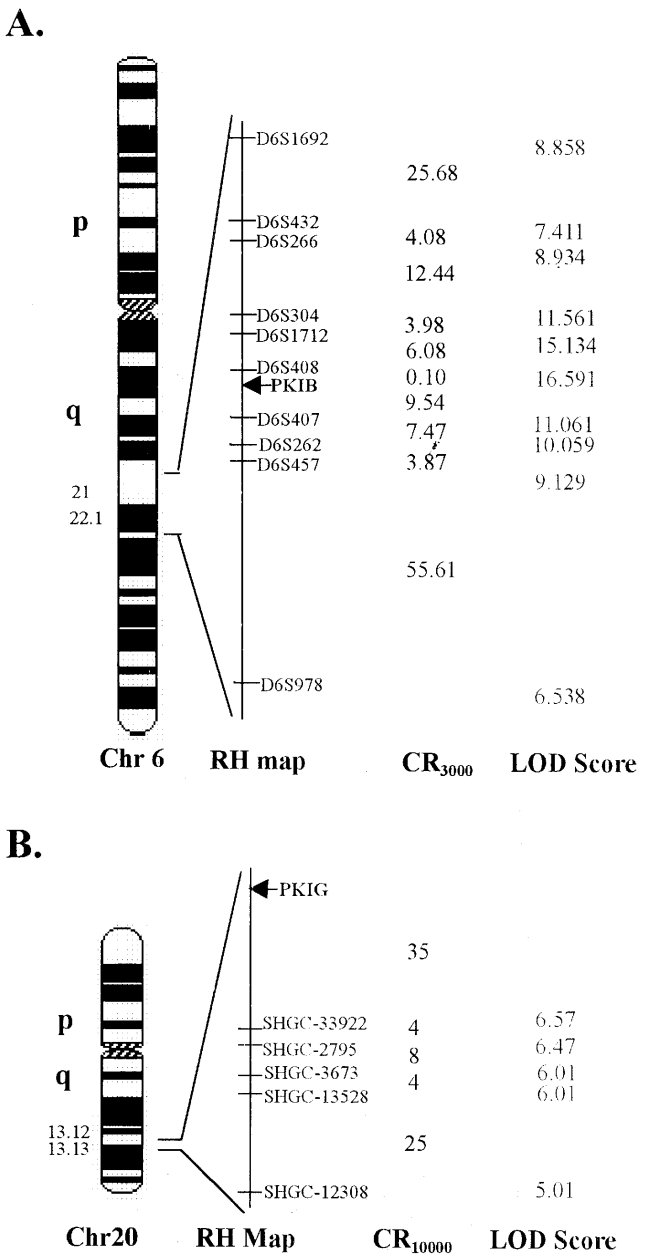

Figure 2 Mapping of *PKIB* (A) and *PKIG* (B)

(**A**) *PKIB* was localized to chromosome 6q21-22.1 between marker D6S408 and D6S407. (**B**) *PKIG* was localized to 20q13.12-13.13 and 35 cR_{10000}, proximal to marker SHGC-33922. Centiray (cR = CR) distances between the markers and the lod score scores associated with each marker are also indicated on the map.

RESULTS AND DISCUSSION

Twenty eight human ESTs with high homology with mouse *PKI*β (GenBank accession number L02241) and 18 human ESTs homologous to mouse *PKI*γ (GenBank accession number U97176) were obtained and assembled into two EST contigs,

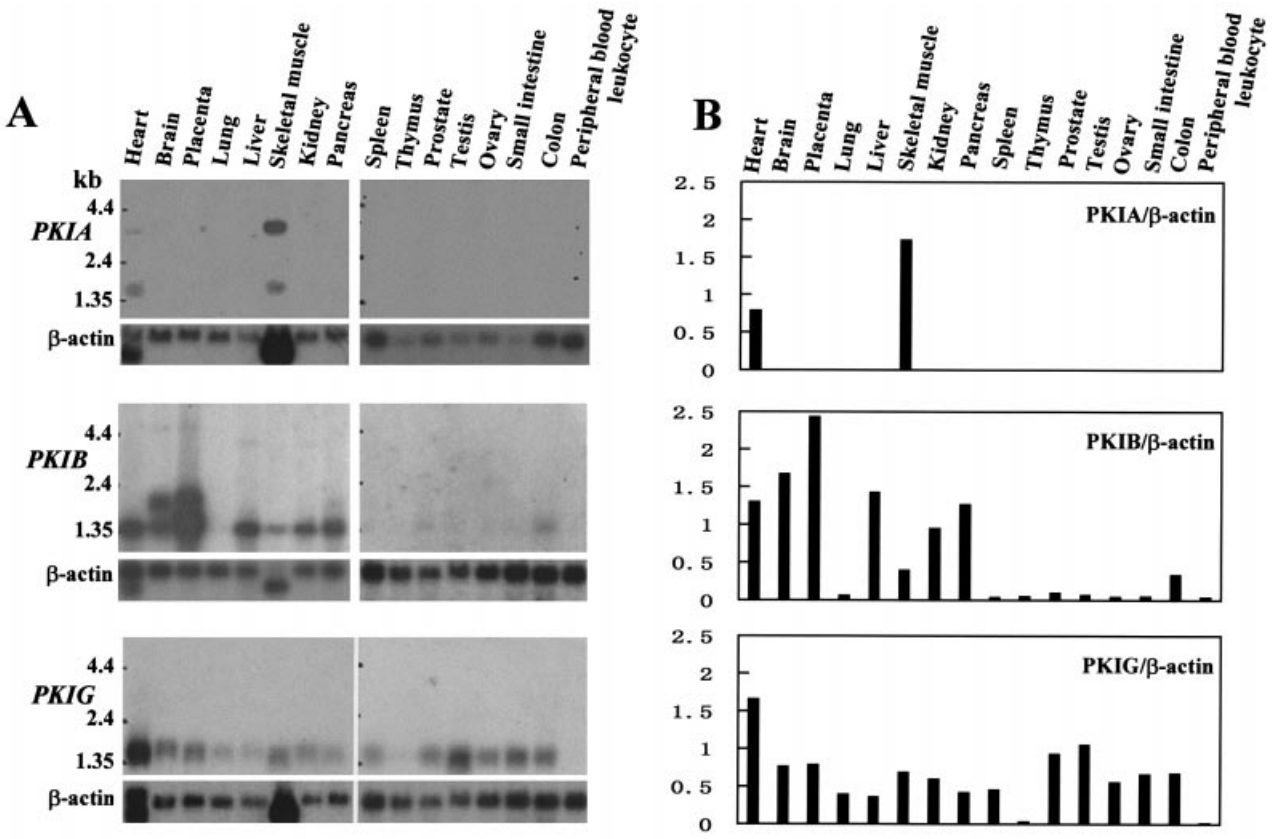

Figure 3 Northern blots of three human *PKI* isoforms in 16 adult human tissues and comparison of tissue expression patterns

(**A**) The membranes (MTNI and MTNII) were hybridized with *PKIA*, *PKIB* and *PKIG*. The Northern blots were also hybridized with the β-actins corresponding to the genes, to allow normalization for differences in loading. (**B**) Ratios of the autoradiographic intensities of the three *PKI* isoforms with those of the corresponding β-actins. The intensities of β-actin expression in heart and skeletal muscle are given as the average of that in brain, placenta, lung, liver, kidney and pancreas. Both long and short forms are included in the intensities of *PKIA* in heart and skeletal muscle, and *PKIB* in brain and placenta. The molecular-size markers (kb) are shown on the left of the panels in (**A**).

each 1.2 kb long. A fragment amplified from a human heart cDNA library was obtained with primers LZB_1 and LZB_2. Another specific fragment amplified from a human testis cDNA library was derived with primers LZG_1 and LZG_2. The two DNA fragments were sequenced and confirmed to be identical with the two contig sequences homologous to mouse *PKI*β and *PKI*γ respectively. These two cDNAs were later respectively named PKIβ (cAMP-dependent, catalytic; *PKIB*) and PKIγ (cAMP-dependent, catalytic; *PKIG*) by the Human Nomenclature Committee. The *PKIB* cDNA contains an open reading frame (nt 258–494) coding for a peptide of 78 amino acids and a 3′-UTR sequence comprising two polyadenylation signals (ATTAAA; nt 681–686 and nt 1164–169) followed by a poly(A)$^+$ tail (Figure 1A). In the 5′-UTR sequence of *PKIB* cDNA there are two in-frame stop codons (TAA; nt 207–209 and nt 237–239) upstream of the start codon. The putative protein shows 70% homology with mouse PKIβ [4] but only 26% identity with the human PKI (i.e. PKIA) reported previously [1]. The *PKIG* cDNA comprises an open reading frame (nt 254–484) which encodes a peptide of 76 amino acids (Figure 1B). The polyadenylation signal (AATAAA; nt 1134–1139) and a poly(A)$^+$ tail are present in the 3′-UTR of human *PKIG*. The deduced protein of *PKIG* shows 90% homology with mouse PKIγ but only 25% identity with the previously reported human PKIA and 18% identity with the PKIB protein.

Notably, the PKI consensus pseudosubstrate site (FxxxxRxGRRNA), which is essential for binding and inhibiting C-subunits of PKA [8–10], exists in PKIB and PKIG (aa 18–29 in PKIB and aa 11–22 in PKIG) (Figures 1A and 1B). Additionally, the leucine-rich nuclear export signal (LxLxLxxLxHy) [11] is highly conserved in PKIB (aa 45–54) (Figure 1A). A segment similar to the nuclear export signal is also present in PKIG (Figure 1B). These conserved sequences found in PKIB and PKIG suggest that they are both novel members of human PKI. To learn more about the structural characteristics of the proteins, the amino acid sequences of PKIs from other organisms were obtained by searching the GenBank database. As expected, the conserved pseudosubstrate site and leucine-rich nuclear export signal motifs (Figure 1C) were defined from the 11 existing PKIs using the MEME program (bits > 3.0), also each of the 11 sequences had an e-value much less than 10 (from 8.1e-8 to 2e-32). The e-value of a sequence is given as the expected number of sequences in a random database of the same size that would match the motifs as well as the sequence does.

The *PKIB* gene was located on a human chromosome using the RH GB4 panel with primers $LZB\text{-}RH_1$ and $LZB\text{-}RH_2$. The results were recorded as follows: 1011100100-1210000100-0000010000-0000100000-1011001000-1010000000-0100100010-1011100100-2010001001-000. The statistical analysis using the Sanger RH server showed the *PKIB* gene was mapped between

marker D6S408 (lod score = 16.6, $cR_{3000} = 0.10$) and D6S407 (lod score = 11.1, $cR_{3000} = 9.54$). These two markers were located between 6q21–22.1 according to the Genome Database comprehensive map. Hence, human *PKIB* was assigned to chromosome 6q21–22.1 (Figure 2A). Similarly, the chromosome localization of human *PKIG* was determined with the G3 panel and primers $LZG\text{-}RH_1$ and $LZG\text{-}RH_2$. The recorded PCR results were 0000000000-0001000000-0000001010-0000010002-0000000000-0000000000-0001010000-0100010000-000 and, after statistical analysis, revealed that *PKIG* was 35 cR_{10000} proximal to SHGC-33922 (lod score = 6.57) and 40 cR_{10000} to SHGC-2795 (lod score = 6.01). The markers were located in chromosome 20q13.12–13.13 according to the GDB database. Therefore human *PKIG* was mapped to 20q13.12–13.13 (Figure 2B).

Northern-blot hybridizations of human *PKIA*, *PKIB* and *PKIG* were performed and their expression patterns were compared. The Northern blot of *PKIA* cDNA, which was obtained by amplifying a human skeletal muscle cDNA library with primers LZA_1 and LZA_2, revealed that *PKIA* was specifically expressed in heart and skeletal muscle as two transcripts of 3.3 kb and 1.5 kb (Figure 3A). Hybridization with the *PKIB* probe detected two transcripts of 1.9 kb and 1.4 kb. The widely expressed 1.4 kb transcript was most abundant in placenta, abundant in brain, heart, liver, and pancreas, moderate in kidney, skeletal muscle and colon, and very weak in lung, spleen, thymus, prostate, testis, ovary, small intestine and peripheral blood leucocytes. The 1.9 kb transcript of *PKIB* was abundant in both placenta and brain. The two transcripts may be attributed to alternative polyadenylation at its two different polyadenylation signal positions. A 1.5 kb transcript of *PKIG* was identified in all tissues examined, with the highest level in heart, lowest in thymus and peripheral blood leucocytes and moderate expression in the other tissues at slightly different levels.

Comparison of tissue expression patterns of the three human PKI isoforms was performed using the ratios of the autoradiographic intensities of *PKI* and β-actin in the 16 tissues tested. As shown in Figure 3(B), both *PKIB* and *PKIG* were widely expressed (all 16 tissues), whereas *PKIA* was only expressed in two tissues, heart and skeletal muscle. The ratio of autoradiographic intensities of PKIs and the corresponding β-actins indicated that PKIB was abundant in placenta (2.43), brain (1.68), liver (1.43), heart (1.30), pancreas (1.27) and kidney (0.95); PKIG was abundant in heart (1.66), testis (1.05), prostate (0.93), placenta (0.79), brain (0.77) and skeletal muscle (0.69). It was also shown (Figure 3B) that heart and skeletal muscle, in which *PKIA* was specifically expressed, were the only tissues in which all three *PKI*s were expressed. In addition, the predominant PKI isoforms were distinct in different tissues. In brain, placenta, liver, kidney and pancreas, PKIB was the predominant PKI isoform. In heart, lung, spleen, prostate, testis, ovary, small intestine and colon, PKIG was the predominant isoform, and PKIA was predominant in skeletal muscle. Interestingly, in thymus and peripheral blood leucocytes the three PKI isoforms were undetectable or showed only trace expression levels. The differences in tissue expression patterns of human *PKIA*, *PKIB* and *PKIG* suggest that they might play different roles in various tissues, individually or co-operatively.

In conclusion, there are at least three PKI isoforms in human tissues, including PKIB, PKIG and the already known PKIA, and two PKI motifs, including the pseudosubstrate site and the nuclear export signal defined with 11 PKIs from six different species. The *PKIB* gene is localized on chromosome 6q21–22.1 and *PKIG* on 20q13.12–13.13. The tissue expression patterns of human *PKIA*, *PKIB* and *PKIG* are obviously different, which might be helpful in the further investigation and understanding of their functions.

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