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Identification of additional members of human G-protein-coupled receptor kinase multigene family

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ABSTRACT **Human neutrophils express several distinct guanine nucleotide binding (G)-protein-coupled receptors that mediate their responsiveness to chemoattractants. Phosphorylation by receptor-specific and second messenger-activated protein kinases is a common mechanism for regulation of G-protein-coupled receptors. To explore the possibility that chemoattractant receptors are regulated by unique receptor kinases, we utilized PCR to identify receptor kinases in human neutrophils. Here, we report the isolation of three G-protein-coupled-receptor-kinase (GPRK)-like sequences termed GPRK5, GPRK6, and GPRK7 in addition to the β-adrenergic receptor kinase (βARK) 1 and 2 isoforms (βARK1 and βARK2). Two, GPRK5 and GPRK6, showed high homology at the amino acid level to the recently identified receptor-kinase-like sequence localized close to the Huntington disease locus. GPRK7 is of interest in that it contains a DLG (Asp-Leu-Gly) amino acid motif of receptor kinases preceded by a DFD (Asp-Phe-Asp) motif. We isolated cDNAs corresponding to GPRK6; the complete sequence shows >66% identity and 81% similarity at the amino acid level to the GPRK from the Huntington disease locus. The GPRK6 cDNA probe hybridizes to two mRNAs of 2.9 and 2.1 kb that were expressed in all the tested human tissues including HL-60 cells and neutrophils. Genomic Southern blot analysis and chromosome mapping showed that GPRK6 hybridizes to two closely related genes located on chromosomes 5 and 13 and are, therefore, distinct from the GPRK located near the Huntington disease locus on chromosome 4. The identification herein of three putative receptor kinases indicates that in addition to βARK and rhodopsin kinase subfamilies, there are other receptor-kinase subfamilies that regulate the broad spectrum of G-protein-coupled receptors.**

Seven-transmembrane-domain-containing receptors transduce a wide variety of signals across the plasma membrane including those triggered by neurotransmitters, hormones, odorants, chemoattractants, and pheromones (1, 2). These receptors are coupled to heterotrimeric guanine nucleotide binding (G) proteins and regulate cellular responses either by activating or inhibiting specific effector enzymes such as adenylyl cyclase and phospholipase C (3). Diverse mechanisms are involved in the regulation of the function of these receptors. These include the rapid events that result in the uncoupling of the receptors from the G proteins (desensitization) and the long-term events that result in receptor sequestration and degradation (1). β-Adrenergic receptors and rhodopsin have been the most extensively studied with respect to these regulatory events (4, 5). β-Adrenergic receptors are rapidly desensitized via phosphorylation by two distinct kinases, namely, the cAMP-dependent protein kinase (heterologous desensitization) (6) and the β-adrenergic receptor kinase (βARK) that phosphorylates only ligand-occupied receptors (homologous desensitization) (7, 8). Biochemical and molecular cloning studies have resulted in the identification of two forms of βARKs (βARK1 and βARK2) and a retina-specific rhodopsin kinase (RK) (8–13). More recently, a cDNA encoding a protein sharing a high degree of homology to the G-protein-coupled receptor kinases (GPRKs) described above has been localized to the Huntington disease locus (14). Homology-based cloning approaches have resulted in the identification of four GPRK-like sequences from *Drosophila* (15).

The main focus of our interest is the regulation of a class of G-protein-coupled receptors that mediate responsiveness to chemoattractants in human phagocytic leukocytes (16, 17). These cells express receptors that respond to distinct peptide chemoattractants like N-formylated peptides (fMet-Leu-Phe), C5a, interleukin 8, and the lipid-derived chemoattractants, platelet-activating factor and leukotriene B4 (16). Recent studies showed that C5a and fMet-Leu-Phe receptors are differentially phosphorylated and desensitized (H. Ali, personal communication). Previous studies have demonstrated the presence of βARK1 in neutrophils (18). The translocation of this enzyme from the cytosol to membrane is effected by platelet-activating factor but not by C5a, suggesting the presence of other receptor kinases. In view of the large number of G-protein-coupled receptors and previous indications for the existence of a large family of receptor-specific kinases (11, 15), we sought to isolate GPRKs, expressed in human neutrophils. Use of PCR to synthesize and amplify fragments of catalytic domains (15, 19) allowed the identification of three GPRK-like sequences. A cDNA for one of these kinases was isolated and characterized as to its expression, genomic organization, and chromosomal localization.‡

MATERIALS AND METHODS

DNA Amplification. Neutrophils were isolated from the blood of a healthy donor as described (20). Total RNA was prepared by guanidinum thiocyanate/phenol/chloroform single-step extraction (21). First-strand cDNA was synthesized using random primers or oligo(dT) and SuperScript reverse transcriptase (BRL) for use as template in the amplification reactions. The sequences of the primers were 5′-TACGAATTCGGN(A/C)GNGGNGGNTT(T/C)GGNGA(A/G)GT-3′ (forward 1), 5′-TACGAATTCGTNTA(T/C)(A/C)GNGA(T/C)(T/C)TNAA(A/G)CC-3′ (forward 2), and 5′-ATCAAGCTTA(C/T)(C/T)TCNGGNGCCAT(A/G)TANCC-3′ (reverse). PCR (22) was done in an Epicomp Tempcycler using Hot Tub DNA polymerase (Amersham) for 40 cycles (1 min at 94°C, 1 min at 55–60°C, and 3 min at 72°C). The annealing temperature in the first cycle was 55°C and was increased by 1°C every cycle and then was fixed at 60°C for cycles 6–40. Amplified DNA from the appropriate size range was purified

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Abbreviations: G protein, guanine nucleotide binding protein; βARK, β-adrenergic receptor kinase; RK, rhodopsin kinase; GPRK, G-protein-coupled receptor kinase.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U00686).

from agarose gels and cloned into the plasmid vector pBluescript. DNA from individual clones were sequenced by the dideoxynucleotide chain-termination method (23) using Sequenase (United States Biochemical).

cDNA Cloning and Nucleic Acid Analysis. DNA probes were labeled by random oligonucleotide priming (BRL). A λgt10 library prepared from dibutyryl-cAMP-treated HL-60 cells (24) was screened with a labeled 493-bp fragment from the GPRK6 PCR clone. Four positive clones ranged in size from 1.8 to 2.55 kb and were subcloned into the plasmid vector pBluescript. The largest of the GPRK6 clones was completely sequenced from a series of nested deletions (25) constructed by using Erase-a-Base (Promega). Fragments from different regions of the GPRK6 cDNA clone were used to probe a human multiple-tissue Northern blot (Clontech) and a Northern blot prepared by us containing the RNAs as indicated.

Genomic Southern Blot Analysis and Chromosomal Localization of GPRK6. Human genomic DNA (Sigma) was digested with various restriction enzymes, electrophoresed on a 0.8% agarose gel, denatured, and transferred to a Hybond nylon membrane (26). Hybridizations were carried out at 65°C in 10% (wt/vol) polyethylene glycol/1.5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/7% (wt/vol) SDS/denatured herring sperm DNA (200 μg/ml) with a 493-bp ^{32}P-labeled fragment from the GPRK6 PCR clone. Filters were washed for two 30-min periods in 2× standard saline citrate (SSC)/0.1% SDS at room temperature and for one 15-min period in 0.2× SSC/0.1% SDS at 65°C and autoradiographed. A panel of human–hamster somatic cell hybrid DNAs digested with *Hin*dIII was obtained from Bios (New Haven, CT) and probed with various fragments from the GPRK6 cDNA. Nylon filters containing *Eco*RI/*Hin*dIII-digested DNAs from monochromosomic cell lines (Coriel Cell Repositories, mapping panel 2) were generously provided by Ram S. Puranam (Department of Medicine, Duke University Medical Center). These filters were also hybridized with a labeled fragment from the 5′ end of GPRK6 cDNA.

RESULTS

Identification of Receptor-Kinase-Like Sequences from Human Neutrophils. Three regions containing conserved amino acid sequences in known receptor kinases and receptor-kinase-like sequences were selected for designing primers for PCR. Two forward primers, one for GRGGFGEV and the other for VYRDLKP amino acid sequence motifs, were synthesized. The reverse primer was derived from the amino acid sequence GYMAPEV/I. PCR was carried out on neutrophil cDNA and the products from the expected size range (500 bp in PCRs with forward primer 1 and reverse primer or 150 bp in the PCRs with forward primer 2 and reverse primer) were purified and directionally cloned into the plasmid vector pBluescript. Of 12 clones sequenced from the 500-bp products, only one contained an open reading frame and showed high homology (94% similarity and 86% identity at the amino acid level) to a receptor-kinase-like sequence (14). None of the other clones contained kinase-like sequences. Of 40 clones sequenced from the 150-bp products, 38 appeared to code for kinase-like sequences based on the presence of the highly conserved tripeptide motif DFG (Asp-Phe-Gly) or the receptor-kinase-associated variation DLG (Asp-Leu-Gly) at the appropriate location (11–15, 27). Nine sequences were represented in the 38 kinase-like clones that were sequenced. Five of them contained the DLG motif and are considered as putative receptor-kinase sequences (Fig. 1). In addition, a highly conserved asparagine and three hydrophobic amino acid residues that occur one amino acid after the proline encoded by the forward primer are all present at the same

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                 *****  *       ********                **
BARK1  VYRDLKP ANILLDEHGH.....VRISDLGLACDFS......KKKP.HASVGTH GYMAPEI
BARK2  VYRDLKP ANILLDEHGH.....ARISDLGLACDFS......KKKP.HASVGTH GYMAPEI
  RK   IYRDLKP ENVLLDDDGN.....VRISDLGLAVELKA.....GQTKTKGYAGTP GFMAPEL
GPRK4  VYRDLKP ENILLDDRGH.....IRISDLGLATEIP......EGQRVRGRVGTV GYMAPEI
GPRK5  VYSDLKP ENILLDDYGH.....IRISDLGLAVKIP......EGDLIRGRVGTV GYMAPEI
GPRK6  VYRDLKP ENILLDDHGH.....IRISDLGLAVHVP......EGQTIKGRVGTV GYMAPEV
GPRK7  VYRDLKP ENILCEHPNQVSPVKICDFDLGSGIKLNGDCSPISTPELLTPCGSA GYMAPEI
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FIG. 1. Alignment of the deduced amino acid sequences of receptor-kinase-like sequences. The deduced amino acid sequences of βARK1, βARK2, GPRK5, GPRK6, and GPRK7 were aligned to maximize homology. The RK sequence and GPRK-like sequence from the Huntington disease locus (GPRK4) as described (12, 14) were included in the comparison. The sequences of the primer region were indicated in boldface type and the direction of amplification is indicated by the arrows. Residues identical in at least six of the seven sequences are indicated by an asterisk. Dots represent gaps in the alignment of sequences.

location in all the sequences. One of these clones was identical to human βARK1 in both nucleotide and amino acid sequence. Another clone showed a single amino acid difference from human βARK1 and bovine βARK2. However, this clone contained several nucleotide differences from human βARK1 (75% identical) but was more closely related to bovine βARK2 at the nucleotide level (85% identical). For this reason we presumed this clone may be the human homologue of bovine βARK2. Fig. 1 shows three receptor-kinase-like sequences, named GPRK5, GPRK6, and GPRK7. RK and the GPRK-like sequence were included to complete the list of all known mammalian receptor-kinase-like sequences in this comparison.

The GPRK6 sequence shown in Fig. 1 was identical in the overlapping regions to the 500-bp PCR clone described above. The GPRK5 also showed high homology to receptor kinases in general (52% identity to βARK1 and 57% identity to RK) and is more closely related to GPRK4 (82% identity) and GPRK6 (80% identity). A search (February 10, 1993) through all the combined protein databases using the BLAST network service of National Center for Biotechnology Information (28), and the GCG sequence analysis package (29) with the three sequences from Fig. 1 revealed homology mainly to other protein-kinase sequences. In all cases the highest homology is to the previously described receptor-kinase sequences followed by other Ser/Thr kinases. Of the three sequences, GPRK7 showed least homology to the known receptor kinases. Nonetheless, it contained the DLG motif and the conserved Glu-Asn-Ile-Leu (ENIL) sequence immediately after the forward primer.

To characterize these clones further, we used the 500-bp fragment from the GPRK6 PCR clone to screen a λgt10 cDNA library and isolated four clones. The largest of these was completely sequenced (Fig. 2). The 2549-bp cDNA sequence contained a single large open reading frame from nt 11 to nt 1642 followed by the TAG stop codon. The 907-bp 3′ untranslated sequence contained stop codons in all reading frames and a polyadenylylation signal AATAAA followed by 65 adenine residues. The long open reading frame codes for a protein of 544 aa containing the three domains typical of receptor kinases. The amino acid sequence similarity within these three domains of GPRK6 with the well-characterized receptor kinases βARK1, RK, and receptor-kinase-like sequences from humans (GPRK4) and *Drosophila* is shown in Fig. 3. The GPRK6 protein is completely collinear with the GPRK4 from methionine through the stop codon of the GPRK4 at aa 501 and showed 66% identity and 81% similarity. The central kinase catalytic domain showed the highest degree of conservation with the kinase domains of known receptor kinases. In this region GPRK6 showed identities of 81% (GPRK4), 75% (*Drosophila* GPRK2), 59% (RK), and 51% (βARK1) (Fig. 3). The numbers in the parenthesis indicate percent similarity to the GPRK6 in that region. While

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GAATTCCGAGATGCTCCAGTTCCCTCACATCAGCCAGTGCGAAGAGCTGCGGCTCAGCCTCGAGCGTGACTATCACAGCCTGTGCGAGCGGCAGCCCATTGGGCGCCTGCTGTTCCGAGA   120
        M  L  Q  F  P  H  I  S  Q  C  E  E  L  R  L  S  L  E  R  D  Y  H  S  L  C  E  R  Q  P  I  G  R  L  L  F  R  E     36

GTTCTGTGCCACGAGGCCGGAGCTGAGCCGCTGCGTCGCCTTCCTGGATGGGGTGGCCGAGTATGAAGTGACCCCGGATGACAAGCGGAAGGCATGTGGGCGGCAGCTAACGCAGAATTT   240
 F  C  A  T  R  P  E  L  S  R  C  V  A  F  L  D  G  V  A  E  Y  E  V  T  P  D  D  K  R  K  A  C  G  R  Q  L  T  Q  N  F     76

TCTGAGCCACACGGGTCCTGACCTCATCCCTGAGGTCCCCCGGCAGCTGGTGACGAACTGCACCCAGCGGCTGGAGCAGGGTCCCTGCAAAGACCTTTTCCAGGAACTCACCCGGCTGAC   360
 L  S  H  T  G  P  D  L  I  P  E  V  P  R  Q  L  V  T  N  C  T  Q  R  L  E  Q  G  P  C  K  D  L  F  Q  E  L  T  R  L  T    116

CCACGAGTACCTGAGCGTGGCCCCTTTTGCCGACTACCTCGACAGCATCTACTTCAACCGTTTCCTGCAGTGGAAGTGGCTGGAAAGGCAGCCAGTGACCAAAAACACCTTCAGGCAATA   480
 H  E  Y  L  S  V  A  P  F  A  D  Y  L  D  S  I  Y  F  N  R  F  L  Q  W  K  W  L  E  R  Q  P  V  T  K  N  T  F  R  Q  Y    156

CCGAGTCCTGGGCAAAGGTGGCTTTGGGGAGGTGTGCGCCTGCCAGGTGCGGGCCACAGGTAAGATGTATGCCTGCAAGAAGCTAGAGAAAAAGCGGATCAAGAAGCGGAAAGGGGAGGC   600
 R  V  L  G  K  G  G  F  G  E  V  C  A  C  Q  V  R  A  T  G  K  M  Y  A  C  K  K  L  E  K  K  R  I  K  K  R  K  G  E  A    196

CATGGCGCTGAACGAGAAGCAGATCCTGGAGAAAGTGAACAGTAGGTTTGTAGTGAGCTTGGCCTACGCCTATGAGACCAAGGACGCGCTGTGCCTGGTGCTGACACTGATGAACGGGGG   720
 M  A  L  N  E  K  Q  I  L  E  K  V  N  S  R  F  V  V  S  L  A  Y  A  Y  E  T  K  D  A  L  C  L  V  L  T  L  M  N  G  G    236

CGACCTCAAGTTCCACATCTACCACATGGGCCAGGCTGGCTTCCCCGAAGCGCGGGCCGTCTTCTACGCCGCCGAGATCTGCTGTGGCCTGGAGGACCTGCACCGGGAGCGCATCGTGTA   840
 D  L  K  F  H  I  Y  H  M  G  Q  A  G  F  P  E  A  R  A  V  F  Y  A  A  E  I  C  C  G  L  E  D  L  H  R  E  R  I  V  Y    276

CAGGGACCTGAAGCCCGAGAACATCTTGCTGGATGACCACGGCCACATCCGCATCTCTGACCTGGGACTAGCTGTGCATGTGCCCGAGGGCCAGACCATCAAAGGGCGTGTGGGCACCGT   960
 R  D  L  K  P  E  N  I  L  L  D  D  H  G  H  I  R  I  S  D  L  G  L  A  V  H  V  P  E  G  Q  T  I  K  G  R  V  G  T  V    316

GGGTTACATGGCTCCGGAGGTGGTGAAGAATGAACGGTACACGTTCAGCCCTGACTGGTGGGCGCTCGGCTGCCTCCTGTACGAGATGATCGCAGGCCAGTCGCCCTTCCAGCAGAGGAA  1080
 G  Y  M  A  P  E  V  V  K  N  E  R  Y  T  F  S  P  D  W  W  A  L  G  C  L  L  Y  E  M  I  A  G  Q  S  P  F  Q  Q  R  K    356

GAAGAAGATCAAGCGGGAGGAGGTGGAGCGGCTGGTGAAGGAGGTCCCCGAGGAGTATTCCGAGCGCTTTTCCCCGCAGGCCCGCTCACTTTGCTCACAGCTCCTCTGCAAGGACCCTGC  1200
 K  K  I  K  R  E  E  V  E  R  L  V  K  E  V  P  E  E  Y  S  E  R  F  S  P  Q  A  R  S  L  C  S  Q  L  L  C  K  D  P  A    396

CGAACGCCTGGGGTGTCGTGGGGGCAGTGCCCGCGAGGTGAAGGAGCACCCCCTCTTTAAGAAGCTGAACTTCAAGCGGCTGGGAGCTGGCATGCTGGAGCCGCCGTTCAAGCCTGACCC  1320
 E  R  L  G  C  R  G  G  S  A  R  E  V  K  E  H  P  L  F  K  K  L  N  F  K  R  L  G  A  G  M  L  E  P  P  F  K  P  D  P    436

CCAGGCCATTTACTGCAAGGATGTTCTGGACATTGAACAGTTCTCTACGGTCAAGGGCGTGGAGCTGGAGCCTACCGACCAGGACTTCTACCAGAAGTTTGCCACAGGCAGTGTGCCCAT  1440
 Q  A  I  Y  C  K  D  V  L  D  I  E  Q  F  S  T  V  K  G  V  E  L  E  P  T  D  Q  D  F  Y  Q  K  F  A  T  G  S  V  P  I    476

CCCCTGGCAGAACGAGATGGTGGAGACCGAGTGCTTCCAAGAGCTGAATGTCTTTGGGCTGGATGGCTCAGTTCCCCCAGACCTGGACTGGAAGGGCCAGCCACCTGCACCTCCTAAAAA  1560
 P  W  Q  N  E  M  V  E  T  E  C  F  Q  E  L  N  V  F  G  L  D  G  S  V  P  P  D  L  D  W  K  G  Q  P  P  A  P  P  K  K    516

GGGACTGCTGCAGAGACTCTTCAGTCGCCAAGATTGCTGTGGAAACTGCAGCGACAGCGAGGAAGAGCTCCCCACCCGCCTCTAGCCCCCAGCCCGAGGCCCCCACCAGCAGTTGGCGGT  1680
 G  L  L  Q  R  L  F  S  R  Q  D  C  C  G  N  C  S  D  S  E  E  E  L  P  T  R  L  *                                        544
AGCAGCTACTCCGAGCGCCGTTTACAGTTTTGCACAGTGATCTTCCCCATTGTCCACTCAAGTCGTGGCCTGGGGAACACAGACGGAGCTGTCCCCAGTGTCCCCCGTCCCTCAGCCCCT  1800
GGCCTGGCTGAGTTTGGCAGGCCTGGGCCATCCCTGGGACAAAGGTGCGTCCCTTCAGCTCTTCTCCGTGGAGCTCGGGGCTTTCTGTATTTATGTATTTGTACGAATGTATATAGCGAC
CAGAGCATTCTTAATTCCCGCCGCAGACCTGGCGCCCCCGCTTGGCTCCTGGGGGCAGCCAGCCCTGGCTGGGAGAGCGGGAGCTGGCAGAGGAGCCACTGCCAAACTCAAGGCTCCTCT  2040
GGCCCAGCTTGGATGGCTGAGGGTGGTCACACCCCTGAGCCTTCAGCACTGTGCTGGCCACCCCGGCCTCTGAGTAAGACTCGTGCCTCCCCCTGCTGCCCTGGGCTCAGGCTGCTACCC
TCTGGGGCCCAAAGCTGTCCCTTCTCAGTGCTTGTCAGCGCTGGGTCTGGGGCCTCTGTATGCCCTAGGCCTGTGCCAAAGTGGCCAGAGATTGGGCTGCCTGTGATACCCATCAGCCCA  2280
CTGCCCCGGCCGGCCCAGATAGGTCTGCCTCTGCCTTCCAGCTCCCACAGCCTGGTCCCTGATACTGGGCTCTGTCCTGCAGACACCTCTTTCAGAAACGCCCAAGCCCAGCCCCTAGGA
GGGGGTGGGGCATCCCTGGTCAACCCTCAAACATTCCGGACTCCCCTCATAACAATAGACACATGTGCCCAGCAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAACGGAATTC                                                                                          2549
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FIG. 2. Nucleotide and the deduced amino acid sequences of the GPRK6 cDNA. The 2.55-kb cDNA was sequenced in both orientations from nested deletions and by using oligonucleotide primers. The potential polyadenylylation signal is underlined. The amino acid sequences that formed the basis for designing the PCR primers are in boldface type and underlined.

the homology was lower in the N- and C-terminal domains, the GPRK6 sequence exhibited significant similarity in these regions to the well-characterized RK and even more similarity to the GPRK4 sequence (Fig. 3). The catalytic domain of the GPRK6 contains all the essential landmarks of Ser/Thr kinases at the same relative positions (27, 30) (Fig. 2). The motif GXGXXG, which forms the ATP binding region (at aa 161–166), Lys-183, Asp-279, and Asn-284, all implicated in ATP binding and catalysis, as well as the tripeptide Ala-Pro-Glu (residues 321–323) are all conserved. One major difference is the Phe → Leu substitution in the invariant tripeptide sequence DFG (27). Indeed, this has become a useful difference to distinguish receptor-kinase-like sequences from other kinase sequences (11–15). Unlike RK, the GPRK6 amino acid sequence did not end with a CAAX box.

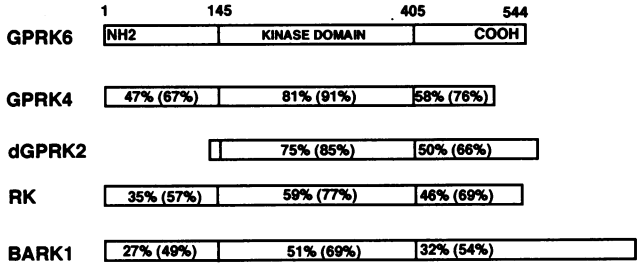

FIG. 3. Comparison of the domain structure of the predicted GPRK6 protein with other related GPRKs. The N-terminal (aa 1–145), kinase catalytic domain (aa 145–405), and C-terminal (aa 405–544) regions of the GPRK6 protein were compared to other GPRKs. The amino acid similarities are in parentheses and identities of each domain are indicated in a linear representation of each of these domains.

A search through the GenBank nucleotide sequences with GPRK6 identified one of the sequences from the 2375 brain cDNAs described by Venter and coworkers (31) (expressed sequence tag EST 00538) as nearly identical. This sequence of 368 bases overlapped with the GPRK6 in the first 280 nt. Analysis of this sequence revealed that 1 nt (G94 in Fig. 2) was missing that would lead to a frame shift in addition to several other unresolved nucleotides. In the first 100 nt of this sequence, another in-frame ATG was present 96 bases upstream of the ATG in the sequence shown in Fig. 2. If this indeed is part of the GPRK6 sequence and the upstream ATG is utilized, it will increase the size of the protein by 32 aa. This will result in a break of exactly 32 aa in the alignment with the highly homologous sequence of GPRK4 but makes the size of the N-terminal domain more comparable to those of βARK1 and RK. Further cDNA cloning and expression analysis are required to resolve which of these ATG codons are utilized.

Genomic Organization and Expression of GPRK6. To determine the genomic organization and complexity of the gene family, genomic Southern blot analysis and chromosome mapping were performed. Fig. 4 shows a Southern blot of human genomic DNA digested with four restriction enzymes and hybridized with a 489-bp fragment from the most conserved region of GPRK6 cDNA (the insert from the GPRK6 PCR clone). The lanes with *Eco*RI- and *Hin*dIII-digested DNA show two bands and the lane with *Bgl* II-digested DNA shows at least three bands. *Eco*RI and *Hin*dIII did not cut within this region of the cDNA. Hybridization and washing at reduced stringency (at 50°C in 2× SSC/0.1% SDS) resulted in at least five bands with each of these enzymes (data not shown). Hybridizations with other regions of cDNA suggested a duplication of the GPRK6 gene, which was confirmed in our attempts to map the GPRK6 gene to human chromosome.

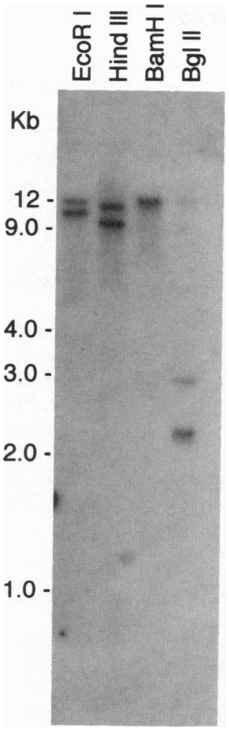

FIG. 4. Genomic Southern blot analysis of GPRK6. Human genomic DNA digested with the indicated enzymes was transferred to Hybond nylon membranes and hybridized with a probe from the GPRK5.

A hamster–human somatic cell hybrid DNA digested with *Hin*dIII was probed for the human GPRK6 gene. In three hybridizations, labeled fragments from the 5′ end (nt 1–430), middle (nt 491–980), and the 3′ end (nt 1875–2485) of the GPRK6 cDNA were used to probe the Southern blot of the somatic cell hybrid panel. As expected from the above results, the complete human DNA showed two bands in all cases. Of 26 hybrids examined, 23 showed hybridization to the smaller of the *Hin*dIII bands. All of these cell lines with the exception of line 423 contained chromosome 5, which is not present in the three nonhybridizing cell lines (data not shown). The larger *Hin*dIII band appeared in 10 of the 26 cell lines. All these cell lines again with the exception of line 423 contained chromosome 13, which is not present in the 16 nonhybridizing cell lines. Although, not identifiable by cytogenetic methods, several sequences known to be located on chromosomes 5 and 13 have been found in cell line 423 (Clark Huckaby, Bios Laboratories, New Haven, CT, personal communication). We have also hybridized Southern blots of *Eco*RI- and *Hin*dIII-digested DNAs of rodent–human cell lines containing single human chromosomes with the 5′ end probe of GPRK6 cDNA. The results confirmed the above observation that the larger *Hin*dIII fragment is located on chromosome 13 and the smaller *Hin*dIII fragment is located on chromosome 5 (data not shown). These results indicate the presence of two GPRK6 genes, one on chromosome 5 and the other on chromosome 13.

To determine the tissue distribution of the GPRK6 transcripts, poly(A)$^+$ RNA from various tissues was analyzed by Northern blot hybridization with the GPRK6 cDNA 5′, middle, and 3′ probes described above. The central (Fig. 5*A*) and the 5′ (data not shown) probes showed identical pattern of hybridization to two mRNAs of 2.1 and 2.9 kb in all the tissues. The 3′-end probe hybridized to only the 2.9-kb mRNA and not to the 2.1-kb mRNA. In addition it also showed hybridization to a large (<8.0 kb) mRNA (data not shown). The placental and skeletal muscle mRNAs showed

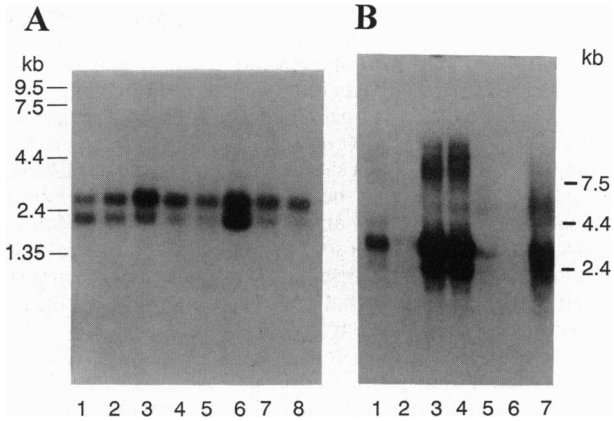

FIG. 5. Tissue distribution of GPRK6 mRNAs. (*A*) A human tissue Northern blot (Clontech) was probed with a 489-bp fragment from the GPRK6 PCR clone. Each lane contains 2.0 μg of poly(A)$^+$ RNA. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. (*B*) Poly(A)$^+$ RNA (2 μg) was separated on a formaldehyde/agarose gel, transferred to nylon membrane, and hybridized (26) to a ^{32}P-labeled 1.6-kb *Eco*RI–*Sac* I fragment from the coding region of the GPRK6 cDNA. Lanes: 1, thymus; 2, HeLa cell; 3, undifferentiated HL-60 cell; 4, dibutyryl-cAMP-differentiated HL-60 cell; 5, U937 cell; 6, no RNA; 7, 20 μg of total RNA from human neutrophils.

stronger hybridization relative to the other tissues. Although the larger transcript is more predominant in most tissues, the smaller mRNA is more abundant in skeletal muscle and heart. In a separate experiment, poly(A)$^+$ RNA from thymus, HeLa, and HL-60 cells and total RNA from human neutrophils were analyzed for GPRK6 expression (Fig. 5*B*). Two mRNAs of the same size as above were detected in all tissues including human neutrophils, the original source of RNA for PCR cloning of GPRK6. The level of expression appears several fold higher in HL-60 cells and human neutrophils relative to HeLa cells, U937 cells, and thymus.

DISCUSSION

Phosphorylation of G-protein-coupled receptors appears to be an important and complex mechanism of cellular regulation. Several types of desensitizations that likely play different physiological roles appear to be mediated by protein kinases. We have identified five receptor-kinase-like cDNA sequences from human neutrophils by using PCR. The tripeptide DFG represents one of the most highly conserved sequence motif in protein kinases (27). In all the receptor kinases that have been characterized the DFG motif is replaced by the tripeptide DLG (11–15). While other clones were identified in the present study only those that contained the DLG tripeptide motif were considered as receptor-kinase-like sequences. The high degree of homology of GPRK5 and GPRK6 to other known GPRKs suggest these clones are very likely to be receptor kinases. In comparison GPRK7 showed very little sequence similarity except for the conserved DLG motif and the 4 aa immediately after the forward primer. A tripeptide DFD motif was found in the place of the conserved DFG in some plant protein-kinase-like sequences (32). In GPRK7, a DFD sequence was present immediately preceding the DLG motif. Such a DFD tripeptide motif was also observed in one of the kinase-like sequences amplified from cellular slime mold *Dictyostelium discoideum* by using the primers described in the present study (unpublished observation). Since *D. discoideum* responds to chemotactic signals as do neutrophils, it will be interesting to see whether these clones are functional homologues.

Although we have not shown that GPRK6 protein has receptor-kinase activity, high homology to RK, which has previously been shown to phosphorylate rhodopsin in a light-dependent manner, suggests that GPRK6 is likely to encode a receptor kinase. All the residues known to be required for kinase activity (27, 28) are conserved in GPRK6. The overall topology of receptor kinases outside of the kinase domain was also preserved. A CAAX box is responsible for membrane localization of the RK (33) and the long C-terminal peptide is responsible for βARK interaction with βγ and translocation to the membrane (34). The absence of both of these characteristics in GPRK6 suggests that alternative mechanisms may be involved in the membrane translocation of GPRK6.

The genomic organization suggests that the GPRK6 locus was duplicated and the two copies of the gene are nonsyntenic. Localization of isoforms to distinct chromosomes has been noted in other instances (35). Indeed, all the receptor kinases thus far identified appear to be located on different chromosomes. βARK1 was localized to chromosome 11 (36) and it was suggested that the highly homologous βARK2 may be located on either chromosome 4 or 7 (13). The localization of GPRK4 near the Huntington disease locus on chromosome 4 (14) and the present identification of two GPRK6 genes on chromosomes 5 and 13 suggest that the members of this family are widely distributed in the genome.

Whereas GPRK4 is expressed predominantly in testis, the GPRK6 transcripts are detected in all human tissues tested including human neutrophils and HL-60 cells, suggesting a broadly distributed function. Based on this pattern of expression, it appears that, while GPRK6 gene product may phosphorylate the chemoattractant receptors that are expressed in the leukocytes, it is likely to phosphorylate other G-protein-coupled receptors. The detection of two mRNAs with the GPRK6 cDNA probe may be explained by their transcription from the two genes located on chromosomes 5 and 13. Alternatively, it is possible that one of the copies of the GPRK6 is a pseudo gene and the two mRNAs are a result of alternative splicing events at the 3′ end of the gene. Further analysis by genomic cloning is required to resolve these possibilities.

The results presented here significantly expand the scope of the GPRK multigene families by adding three sequences to the four already known. It also defines a subfamily of receptor kinases that include GPRK4, GPRK5, and GPRK6 from humans and dGPRK2 from *Drosophila*. Thus receptor regulation by phosphorylation may be modulated differentially by receptor-kinase subfamilies in a far more complex and class-specific manner than previously recognized.

Note Added in Proof. After submission of this manuscript Kunapuli and Benovic (37) reported the cloning and expression of a G-protein-coupled receptor kinase (GRK5) that is identical to the short GPRK5 sequence described in this paper.

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