Identification and Characterization of a Novel Related Adhesion Focal Tyrosine Kinase (RAFTK) from Megakaryocytes and Brain*

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We have isolated a cDNA encoding a novel human intracytoplasmic tyrosine kinase, termed RAFTK (for a related adhesion focal tyrosine kinase). In addition, we have cloned and characterized the murine homolog of the human RAFTK cDNA. Comparison of the deduced amino acid sequences of human RAFTK and murine Raftk cDNAs revealed 95% homology, indicating that RAFTK is highly conserved between these species. The RAFTK cDNA clone, encoding a polypeptide of 1009 amino acids, has closest homology (48% identity, 65% similarity) to the focal adhesion kinase (pp125FAK). Comparison of the deduced amino acid sequences also indicates that RAFTK, like pp125FAK, lacks a transmembrane region, myristylation sites, and SH2 and SH3 domains. In addition, like pp125FAK, RAFTK contains a kinase domain flanked by large N-terminal (426 residues) and C-terminal (331 residues) domains, and the C-terminal region contains a predicted proline-rich stretch of residues. In fetal tissues, RAFTK expression was abundant in brain, and low levels were observed in lung and liver. In adult tissues, it was less restricted, indicating that RAFTK expression is developmentally up-regulated. Expression of RAFTK was also observed in human CD34+ marrow cells, primary bone marrow megakaryocytes, platelets, and various areas of brain. The human RAFTK gene was assigned to human chromosome 8 using genomic DNAs from human/rodent somatic cell hybrid lines. The mouse Raftk gene was mapped to chromosome 14, closely linked to gonadotropin-releasing hormone. Using specific antibodies for RAFTK, a ~123-kDa protein from the human megakaryocytic CMK cell line was immunoprecipitated. Treatment of the megakaryocytic CMK cells with thrombin caused a rapid induction of tyrosine phosphorylation of RAFTK protein. The structural features of RAFTK suggest that it is a member of the focal adhesion kinase gene family and may participate in signal transduction in human megakaryocytes and brain as well as in other cell types.

Protein-tyrosine kinases participate in a variety of signal transduction pathways that modulate cell growth and differen-

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This paper is dedicated to Ronald Ansini for his friendship and support for our research program.

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signal transduction and may have a role in cell growth and differentiation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Chemical reagents were purchased from Sigma. Restriction endonucleases, modifying enzymes, and terminal deoxynucleotidyltransferase were purchased from Pharmacia Biotech Inc. and New England Biolabs Inc. (Beverly, MA). The primers for polymerase chain reaction (PCR), 5' RNA PCR, and sequencing were synthesized by an automatic DNA synthesizer (Applied Biosystems Inc., Model 394). The PCR and RNA PCR reagents were obtained from Perkin-Elmer, and random-primer labeling kits were obtained from Stratagene (La Jolla, CA). Manual and automated sequencing kits were obtained from U. S. Biochemical Corp. and Pharmacia Biotech Inc., respectively. Automation of sequencing was performed using Pharmacia’s automated fluorescent sequencer. Monoclonal antibody 2A7 against pp125sint protein was kindly obtained from Dr. J. Thomas Parsons (Department of Microbiology, University of Virginia School of Medicine, Charlottesville, VA). Monoclonal antibody PY-20 directed against Tyr(P) was obtained from ICN (Costa Mesa, CA).

**Cells—**Human marrow megakaryocytes were isolated by a method employing immunomagnetic beads using an anti-human glycoprotein IIIa monoclonal antibody as described previously (33, 34). CD34-bearing marrow progenitor cells were purified by heparinized bone marrow aspirates using immunomagnetic beads coated with an anti-CD34 monoclonal antibody as described previously (34). The CMK cell line, provided by Dr. T. Sato and derived from a child with megakaryoblastic leukemia, was from stocks of cells of the megakaryocytic line CMK. The CMK cell line was cultured in RPMI 1640 medium with 10% fetal calf serum. Additional permanent human megakaryocytic cell lines were studied. DAMI cells were obtained from Dr. S. Greenberg (Brigham and Women’s Hospital, Boston, MA); Mo7e and erythroid megakaryocytic HEL cells were obtained from Dr. L. Zon (Children’s Hospital, Boston, MA). Each cell line was cultured as described previously (34, 36, 37). Other permanent human cell lines such as Ramos (human B-cells) were obtained from the American Type Culture Collection and maintained in liquid culture according to the specifications in the catalog. Human platelets were isolated by gel filtration from freshly drawn blood anticoagulated with 0.15 volume of NIH formula A citrate/dextran solution supplemented with 1 μM prostaglandin E1, as described previously (38).

**DNA Amplification and Cloning—**Total RNA derived from CMK cells was prepared by a standard protocol of lysis in guanidinium isothiocyanate followed by cesium chloride gradient centrifugation (38). Protein tyrosine kinase sequences were amplified with degenerate oligonucleotide primers synthesized previously (38). Briefly, total RNA (10 μg) was used as a template for synthesis of cDNA. The PTK3 oligonucleotide DS-DS(A/G)TC(A/C/G/T)ACCTAAA(A/C/G/T)(C/G)- (T/A)(A/G)(T/A)(A/C/G/T)CC-3' was designed in our laboratory and was used as a primer. PCR was performed on one-fourth of the cDNA synthesis reaction mixture (original volume of 20 μl) using the PTK1 DL1 primer (5'-CT(A/G)TC(A/C/G/T)ACCTAAA(A/C/G/T)(C/G)-(T/A)(A/G)(T/A)(A/C/G/T)) and PT2K WMAPE (5'-GGTACC(T/C)TC(G/C/A)GG(A/C/G/T)-(A/G)CC(T/A)(T/A)(T/A)-3') using the PTK1 DL1 as a primer. PCR products were cloned into pBSK, and thereafter sequenced. Nucleotide sequences were determined by the automated laser fluorescent DNA sequencing using Autosearch (Pharmacia Biotech Inc.) and by manual sequencing using the Sequenase kit.

**Chromosomal Localization of the Human RAFTK Gene—**Genomic DNAs from the NIGMS hybrid mapping panels 1 and 2 were obtained from NIGMS Genomic Cell Mutant Repository (Coriell Cell Institute for Medical Research, Camden, N J.). In addition, both mapping panels included DNA samples isolated from human and rodent parental cell lines (mouse and Chinese hamster). Approximately 5 μg of human, hamster, and mouse genomic DNAs were digested with BamHI, HindIII, and PstI to find a suitable restriction fragment length polymorphism (RFLP) or unique genomic fragment for use in mapping. Subsequently, genomic DNAs from each panel were cut with BamHI. Southern blots were probed with a 1.4-kb human RAFTK cDNA, and hybridizations were carried out as described previously (40, 41). Hybrid blots were scored for the appropriate human-specific restriction endonuclease fragment on the autoradiographs. The results were compared with the chromosome contents of the hybrid cell lines, and the concordance between restriction fragments and specific chromosome content was used to establish the localization of the human RAFTK.

**Backcross Mapping of the Mouse Raftk Gene—**Genomic DNAs from C57BL/6J, Mus spretus, and a (C57BL/6J × M. spretus) M. spretus BSS-type backcross DNA panel were obtained from The Jackson Laboratory (Bar Harbor, Maine) (40). Southern blots and hybridizations were performed as described previously (41). Approximately 5 μg of C57BL/6J and M. spretus genomic DNAs were digested with 29 different restriction enzymes to identify a potential RFLP genetic marker. The Southern blots were probed with a 1.4-kb human RAFTK cDNA fragment labeled with [α-32P]dCTP using random-primer cDNA labeling. Hybridization to nylon filters (MSI) was performed in 50% formamide, 6 × SSC, 10 μl sodium phosphate, 5 × Denhardt’s solution, 0.1% SDS, and 1 mg/ml herring sperm DNA (Boehringer Mannheim) at 43°C overnight. The filters were washed at room temperature in 2 × SSC, 1% SDS and then in 0.2 × SSC, 0.1% SDS at 63°C three times for 30 min; probed developed (Stratagene) using a UV-cross-linker (Stratagene Stratalinker); and exposed to Kodak X-OMAT AR film (Eastman Kodak Co.). Twelve clones were isolated and processed. Plasmid DNA was prepared using Exassist helper phage and the Xor system according to the manufacturer’s instructions (Stratagene). Of these 12 clones, two were sequenced on both strands. A human CMK phorbol 12-myristate 13-acetate cDNA library oligo(dT)17 (38) recombinants screening) (36) in xg10 vector was screened with the 32P-labeled J3 fragment. Four clones were isolated; the recombine DNAs of two positive phages were digested with EcoRI, and the cDNA insert was subcloned into pBSK (Stratagene) and thereafter sequenced.

A 346-bp probe was prepared from the 5'-end of one of the CMK cDNA clones (termed 2-1) and used to screen the human brain (hippocampus) cDNA library. Twelve clones were isolated, and two clones were sequenced on both strands. In addition, a 248-bp probe was prepared from the 5'-end of one of the clones (termed 4C), and the human hippocampus cDNA library was rescreened. Twelve clones were identified and isolated, and of these, one clone (termed 38) was sequenced on both strands.

**The mouse brain cDNA library (catalog No. ML1042b, clone TECH, Palo Alto, CA) in λg11 vector was screened (5 × 106 recombinants screening) using the 381-bp 5'-Kpn1 fragment or the 764-bp Apal 3'-fragment of human RAFTK cDNA as a probe, and the filters were hybridized and washed under high stringency conditions. Six clones were isolated. The DNA was isolated as described previously (38), subcloned into pBSK, and thereafter sequenced. Nucleotide sequences were determined by the automated laser fluorescent DNA sequencing using Autoread (Pharmacia Biotech Inc.) and by manual sequencing using the Sequenase kit.

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1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); RFLP, restriction fragment length polymorphism; RI, recombinant inbred; FAK, focal adhesion kinase; GST, glutathione S-transferase.
Northern Blot Analysis—Total RNA was prepared by a standard protocol of lysis in guanidinium isothiocyanate followed by cesium chloride gradient centrifugation (38). The human adult and fetal tissue Northern blots, the brain regions, and the human tissue II blots were obtained from CLONTECH. Hybridization was carried out according to the manufacturer’s instructions. Each RNA blot was probed with a 1460-bp insert of RAFTK cDNA cloned into the human adult and fetal marrow cells (106 cells), and bone marrow megakaryocytes (106 cells) amplified by PCR using specific RAFTK primers as described previously (31). The sequence of the RAFTK upstream primer was 5’-GGGCGTGCCTGAGACTC-3’ (positions 2958–2977) (see Fig. 1B). The nucleotide sequence of the RAFTK downstream primer was 5’-CTGCTAGAGATGTCC-3’ (positions 3084–3103) (see Fig. 1B). The sequence of the FAK upstream primer was 5’-AAAGCTGCTACGAGATGTCC-3’ (positions 2922–2932). The nucleotide sequence of the downstream primer of the RAFTK cDNA was 5’-CTGGTGTGCGTTGAGCTTGAAG-3’ (positions 2417–2438) (43). The sequence of the actin upstream primer was 5’-ACACACCCATGTCGAGCTGC-3’. The nucleotide sequence of the downstream primer of the human actin was 5’-GTGCTACTACTGCTGCTGTTACACATGC-3’ (CLONTECH). The PCR products were electrophoresed on a 1.5% agarose gel, denatured, neutralized, transferred to filters, and vacuum-blotted. The probes used were the RAFTK, FAK, and actin gene-specific probes, which were labeled by random priming as described above. Prehybridization and hybridization were carried out as described previously (31).

Protein Analysis—Metabolic labeling, immunoprecipitation, and Western blot analysis were performed in CMK cells as described previously (44–47). For immunoblot analysis, total cell lysates of CMK cells untreated or stimulated with thrombin (1 or 2 units/ml as indicated), ChromoLog Corp., Havertown, PA) for 5 min were prepared as described previously (45). Relative protein concentrations were determined with a Betascope 603 blot analyzer (Betagen, Mountain View, CA). The same blot was assessed for the presence of the actin or glyceraldehyde-3-phosphate dehydrogenase-specific probes.

PCR Blots—cDNA was prepared from platelets (10 × 10^7), CD34− marrow cells (10^6 cells), and bone marrow megakaryocytes (10^6 cells) amplified by PCR using specific RAFTK primers as described previously (31). The sequence of the RAFTK upstream primer was 5’-GGGCGGCGTGCGCTGAGACTC-3’ (positions 2958–2977) (see Fig. 1B). The nucleotide sequence of the RAFTK downstream primer was 5’-CTGCTAGAGATGTCC-3’ (positions 3084–3103) (see Fig. 1B). The sequence of the FAK upstream primer was 5’-AAAGCTGCTACGAGATGTCC-3’ (positions 2922–2932). The nucleotide sequence of the downstream primer of the RAFTK cDNA was 5’-CTGGTGTGCGTTGAGCTTGAAG-3’ (positions 2417–2438) (43). The sequence of the actin upstream primer was 5’-ACACACCCATGTCGAGCTGC-3’. The nucleotide sequence of the downstream primer of the human actin was 5’-GTGCTACTACTGCTGCTGTTACACATGC-3’ (CLONTECH). The PCR products were electrophoresed on a 1.5% agarose gel, denatured, neutralized, transferred to filters, and vacuum-blotted. The probes used were the RAFTK, FAK, and actin gene-specific probes, which were labeled by random priming as described above. Prehybridization and hybridization were carried out as described previously (31).

RNA binding sites were obtained of the coding region of RAFTK. The nucleotide sequence and the deduced amino acid sequence of the encoded protein with the National Biomedical Research Foundation and GenBankTM Data Banks revealed that this cDNA encoded a tyrosine kinase related to pp125FAK. The predicted amino acid sequence of pp125RAFTK contains the structural motifs common to all protein kinases, including the putative ATP-binding site (Gly432-Xaa-Gly434-Xaa-Gly437) and 3 residues that are predicted to interact with the γ-phosphate group of the bound ATP molecule (at amino acids 402, 529, and 655). In addition, RAFTK contains two peptide sequences that are highly conserved among protein-tyrosine kinases (Asp349, Ile550, Ala551, Val552, Arg553, Asn554, and Pro588-Ile589-Lys590; Trp591-Met592). Interestingly, like chicken pp125RAFTK, the C-terminal region of RAFTK contains a proline-rich stretch (residues 690–767) in which the proline content exceeds 20%. A unique domain is found at the N terminus of RAFTK (amino acids 1–39) (Fig. 2 and Fig. 3). This region is the most divergent among various protein-tyrosine kinases and may be involved in cellular localization and/or interaction with other cellular proteins. Like pp125RAFTK, RAFTK does not contain SH2 or SH3 domains. The kinase domain (amino acids 427–679) of RAFTK shares 60% identical homology with mouse pp125RAFTK, 54% with human pp125RAFTK, and 36% with Src (Fig. 3). The kinase domain consists primarily of the catalytic domain including the putative ATP-binding site (amino acids 432–437). RAFTK shares 42% homology in the N-terminal domain and ∼39% in the C-terminal domain with mouse pp125RAFTK. The overall amino acid homology of RAFTK is 48% identity (65% similarity) to mouse pp125RAFTK.
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Fig. 1. A, schematic representation and restriction enzyme map of the RAFTK cDNA. The various cDNA clones, obtained from the human hippocampus cDNA library (in λZAPII vector) and the CMK phorbol 12-myristate 13-acetate cDNA library (in λgt10 vector), are shown as indicated. Restriction enzyme sites are indicated along the length of the cDNA.

B, nucleotide and deduced amino acid sequences of the RAFTK cDNA clone representing the full-length cDNA. Nucleotide numbers are shown on the left. The amino acid numbers are shown on the right. The putative initiation codon at nucleotides 294–296 is shown in boldface type. The catalytic domain is boxed. The ATP-binding site is underlined, and the putative phosphorylation sites are encircled. The asterisk refers to the stop codon.

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A random- and oligo(dT)-primed mouse adult brain cDNA library was screened under conditions of high stringency for the full-length mouse RAFTK cDNA using the 5′- and 3′-fragments of human RAFTK cDNA as probes. Four clones were isolated; two of these clones were sequenced in both directions, and additional clones were partially sequenced. Sequence analysis of these clones revealed identical sequences. The 4.5-kb full-length cDNA has an open reading frame of 1009 amino acid...
residues and possesses 95.6% identical homology to the human RAFTK gene (Fig. 2).

Chromosomal Localization of the Human RAFTK Gene—Hamster, human, and mouse DNAs were digested with BamHI, HindIII, and PstI to identify a specific RFLP pattern for the RAFTK gene in each species. Southern blots were probed with a 1.4-kb human RAFTK cDNA. Unique 16.5- and 14.5-kb BamHI fragments for RAFTK were identified in human DNA from the parental cell lines used to prepare human/rodent cell hybrids (Fig. 4). DNAs from the parental and somatic hybrid cell lines in mapping panel 2 were digested with BamHI, Southern-blotted, and probed. Analysis indicated that the human-specific BamHI pattern was observed in cell line 8, which contains human chromosome 8 (Fig. 4). A fainter signal was also observed for the human-specific BamHI pattern in hybrid cell line 20 (Fig. 4), which contains intact human chromosome 20, but also carries a gene from human chromosome 8 (NEFL (neurofilament light polypeptide), 8p21) as determined by Southern blot hybridization (Coriel Cell Institute for Medical Research). All other hybrid cell lines were negative for the human-specific BamHI RFLP. Additionally, when the 1.4-kb human RAFTK cDNA was used to probe Coriel mapping panel 1, the human-specific fragment was detected in all hybrids containing >4% of human chromosome 8 and was absent in every hybrid that lacked chromosome 8 (Table I).

Chromosomal Localization of the Mouse Raftk Gene—Southern blots of C57BL/6J and M. spreitus DNAs were digested with 29 different restriction enzymes and probed with a 1.4-kb hu-
man RAFTK cDNA. A BamHI RFLP was detected (Fig. 5A). The alleles for this BamHI RFLP consist of 8.6- and 5.2-kb genomic DNA bands, characteristic of C57BL/6J, and 15.5- and 6.7-kb bands, which are found in M. spreitus. These alleles were characterized in 87 DNAs from the C57BL/6J × M. spreitus backcross panel. Results of the haplotype analysis from this mapping data indicate that the RAFTK gene colocalizes with D14Bir10 (DNA segment Birkenmeier 10) and is linked to Nfl (neurofilament light polypeptide) on mouse chromosome 14 (Fig. 5B). The RAFTK locus mapped between Xmv19 (xenotropic MCF leukemia virus-19) and Nfl, and the calculated map distances for these loci are as follows: Xmv19, 7.1 ± 5.3 cM, RAFTK, 3.5 ± 2.0 cM, Nfl.

The position of RAFTK on mouse chromosome 14 was confirmed by determining the segregation of a SacI RFLP for RAFTK DNAs from BxD RI lines. The SacI RFLP for RAFTK was indicated by the presence of a 16.5-kb genomic DNA band in C57BL/6J or a 6.2-kb fragment in DBA/2 (Fig. 6A). These alleles were characterized for 26 DNAs from the BxD RI line (Fig. 6B). The strain distribution patterns of the RAFTK loci were characterized for 26 DNAs from the BxD RI line (Fig. 6B). Perfect concordance was observed with the BxD strain distribution pattern for the Nfl locus, indicating linkage of <1-map unit distance from RAFTK Nfl (51). These mapping data place RAFTK distal to Nfl and are a contradiction to the backcross data. However, backcross data are not as accurate as RI data since backcross mice were derived from an interspecies cross.

Expression of RAFTK in Tissues and Cell Lines—A specific RAFTK probe was designed (nucleotides 2958–3103). This sequence is present in RAFTK and not in human pp125AK. This probe was used for hybridization of the cDNA probe to a Northern blot of poly(A+) RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. While heart and skeletal muscle RNA samples were negative for RAFTK, a single mRNA was observed in all other tissues, with the highest levels expressed in brain (Fig. 7B). To further characterize the distribution of RAFTK expression in other human tissues, Northern blot analysis of spleen, thymus, prostate, testis, ovary, intestine, colon, and peripheral blood leukocytes revealed a high level of expression of RAFTK in thymus, spleen, and peripheral blood leukocytes (Fig. 7C). Northern blot analysis of different human brain regions (amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus, and thalamus) revealed that the highest level of expression of RAFTK was in the amygdala and hippocampus (Fig. 8). A lower level of expression was observed in other brain regions, with the exception of the corpus callosum and substantia nigra, where there was no detectable signal. These results indicate that the brain has abundant expression of RAFTK, especially in the amygdala and hippocampus.

Expression of RAFTK was observed in several megakaryocytic cell lines such as CMK, Mo7e, HEL, and DAMI (data not shown). In addition, expression of RAFTK was detected in Ramos, FHS, and HeLa cells, but a low level of expression was detected in J urkat, Hep3B, and CCL75 cells (data not shown). Using PCR techniques, expression of RAFTK was also found in primary bone marrow megakaryocytes, blood platelets, and CD34+ marrow progenitor cells. Interestingly, it appears that

![Mapping of RAFTK in humans to chromosome 8 using human RAFTK cDNA.](http://www.jbc.org/)

![Mapping of RAFTK in humans to chromosome 8 using human RAFTK cDNA.](http://www.jbc.org/)

### Table 1

Concordance of the human RAFTK gene with human chromosomes in human/rodent somatic cell hybrids

| Hybrid clone | RAFTK | Human chromosome |
|--------------|-------|------------------|
| GM09925      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09926      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09927      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09928      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09929      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09930A     | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09931      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09932      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09933      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09934      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09935A     | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09936      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09937      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09938      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM09940      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM10324      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM10567      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |
| GM10611      | +     | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y |

| % of concordant hybrids | 61 61 61 67 50 83 72 89 33 67 56 56 61 6 83 44 78 61 78 56 44 65 39 33 |

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polyclonal antibodies (R-4250) do not cross-react with the GST-RAFTK C terminus fusion protein. These results indicate that RAFTK might be antigenically different from FAK. Furthermore, FAK immunoprecipitated by monoclonal antibody 2A7 from megakaryocytes was not recognized by polyclonal antiserum 4250 (R-4250). Similarly, RAFTK immunoprecipitates (Fig. 10A) were not recognized by monoclonal antibody 2A7. Taken together, these data indicate that RAFTK is a protein-tyrosine kinase that can induce its tyrosine phosphorylation.

**DISCUSSION**

The method of PCR cloning has been successfully employed by many laboratories to identify novel members of the protein-tyrosine kinase family. Using this strategy, we have identified a novel intracytoplasmic tyrosine kinase in human megakaryocytic cells that we have termed RAFTK. Sequence analysis of RAFTK revealed a highly conserved kinase domain that is similar to that of FAK and Gnrh. RAFTK does not appear to have any effects that have been described for FAK or Gnrh, indicating that RAFTK and FAKB are different proteins.

The chicken, human, and mouse focal adhesion kinases have restricted expression in various normal tissues, indicating that their expression is up-regulated during development. The level of expression of RAFTK mRNA is similar to that of FAK in CD34+ cells, which is higher than that of FAK in bone marrow megakaryocytes. In platelets, the level of expression of RAFTK mRNA is lower than that of FAK, as observed by PCR. RAFTK mRNA expression in bone marrow megakaryocytes is higher than that in CD34+ cells (Fig. 9). Taken together, these results indicate that RAFTK is abundantly expressed in brain and hematopoietic cells. The restricted expression observed in fetal versus adult tissues indicates that its expression is up-regulated during development.

**Generation of Specific Antibodies for RAFTK and Detection of RAFTK Protein**—The GST-RAFTK C terminus fusion protein (residues 681-1009) was chosen for rabbit immunizations in order to obtain specific antibodies for RAFTK protein. These polyclonal antibodies (R-4250) do not cross-react with pp125FAK. The monoclonal antibody 2A7 against FAK does not cross-react with the GST-RAFTK C terminus fusion protein, indicating that RAFTK might be antigenically different from FAK. Furthermore, FAK immunoprecipitated by monoclonal antibody 2A7 from megakaryocytes was not recognized by polyclonal antiserum 4250 (R-4250). Similarly, RAFTK immunoprecipitated by R-4250 also was not recognized by monoclonal antibody 2A7. Taken together, these data indicate that FAK and RAFTK are distinguishable antigenically while being related members of the FAK family.

The specificity of this antiserum was examined by immunoprecipitation. The CMK cell line was metabolically labeled with [35S]methionine, and extracts were immunoprecipitated with anti-RAFTK antiserum. A major protein species of ~123 kDa was detected in CMK cells (Fig. 10A). A similar species was observed in other human megakaryocytic cell lines such as DAM1 (data not shown). This band was not observed when normal rabbit serum or preimmune rabbit serum was used for immunoprecipitation. Incubation of R-4250 with 1 or 10 μg of the GST-RAFTK C terminus fusion protein abolished the appearance of the ~123-kDa protein, while incubation with 10 μg of the GST-MATK (where MATK is megakaryocyte tyrosine kinase) SH2 domain fusion protein did not have any effects. These results indicate that R-4250 polyclonal antibodies specifically recognize RAFTK protein of ~123 kDa in size. Furthermore, thrombin (1 unit/ml) stimulated a rapid increase in the amount of RAFTK protein immunoreactivity in anti-Tyr(P) immunoprecipitates (Fig. 10B). These results indicate that RAFTK is a protein-tyrosine kinase that can induce its tyrosine phosphorylation.
We have cloned the murine homolog of the human RAFTK cDNA. The sequence identity between the human RAFTK and murine Raftk cDNAs is 90% at the nucleotide level and 95.6% at the predicted amino acid level. In the kinase domain, 98.5% of the amino acids are identical (Fig. 2). Therefore, the RAFTK gene is highly conserved in human and rodent, again suggesting an important role in cell signaling functions. RAFTK has an insertion of an additional 4 amino acids between positions 76 and 81 (Gly76-Arg77-Ile78-Gly79) compared with chicken, murine, and human pp125FAK sequences (13, 14, 19). Amino acids corresponding to positions 292–320 of human pp125FAK and amino acids corresponding to positions 850–864 and 901–926 of chicken pp125FAK are absent in the predicted RAFTK protein. Interestingly, like chicken pp125FAK, the C terminus region of human RAFTK and mouse Raftk contains a proline-rich stretch (residues 690–767). It has been shown that proteins containing proline-rich peptide motifs (such as Shc, p62, and ribonucleoprotein K) could serve as SH3 domain ligands and that the binding of these proteins to the Src SH3 domain was inhibited with a proline-rich peptide ligand (54). Furthermore, the predicted RAFTK protein, like the pp125FAK protein, displays several unique features among the known tyrosine kinases. The primary sequence of RAFTK does not contain a signal peptide or a membrane-spanning region, and the protein is therefore presumed to be located in the cytoplasm. RAFTK lacks SH2 and SH3 domains, which are structural elements involved in protein-protein interactions (2, 47, 55–57), and does not exhibit significant homology to any known protein-tyrosine kinase beyond pp125FAK outside of the catalytic domain (Figs. 2 and 3).
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Lack of SH2 and SH3 domains suggests that other regions within RAFTK protein are important for protein interaction and targeting. In the case of the pp125FAK protein, it has been demonstrated by structural-functional analysis that 159 amino acids within the C-terminal noncatalytic domain of pp125FAK including Tyr397 did not abolish the kinase activity of pp125FAK (59). Moreover, there is conservation of several tyrosine residues between RAFTK and pp125FAK (Figs. 2 and 3), including Tyr397, which has been shown to be the major site of tyrosine phosphorylation in pp125FAK protein (25).

RAFTK-specific mRNA expression was observed in human fetal tissues, being most abundant in brain (predominantly in the amygdala and hippocampus regions), and appeared to be developmentally up-regulated as demonstrated in the pattern of adult tissue expression (Figs. 7 A–C and 8). Within the hematopoietic system, in addition to peripheral blood leukocytes, a high level of specific mRNA expression of RAFTK was detected in B-cells and various megakaryocytic cell lines (data not shown). By using PCR, the specific mRNA expression of RAFTK was also detected in CD34+ primary bone marrow progenitor cells, primary bone marrow megakaryocytes, and platelets (Fig. 9).

RAFTK is phosphorylated after thrombin treatment of CMK cells. Interestingly, FAK protein was also found phosphorylated on tyrosine after thrombin or collagen treatment of platelets (18). There is considerable homology in the thrombin receptors and considerable signal similarities in transduction mechanisms between platelets and megakaryocytes (60). Furthermore, bone marrow megakaryocytes in liquid culture stimulated with thrombin for 5 min revealed dramatic morphological changes reminiscent of those found in platelets, including shape change and organelle centralization that involved immature as well as mature cells (61). Megakaryocytes were also able to secrete α-granule proteins in the dilated cisternae of the demarcation membrane system (61).

The human RAFTK gene was found on chromosome 8 using DNAs from the somatic cell hybrid lines (Fig. 4). The signal observed in cell line 20 in mapping panel 2 suggested that a fragment of chromosome 8 is in the chromosome 20 cell line. Indeed, although cell line 20 contained the human NEFL gene, there was no evidence of chromosome 20 or a fragment of chromosome 20 in cell line 8 (Coriell Cell Institute for Medical Research). Indeed, the localization of RAFTK to chromosome 8 was confirmed using mapping panel 1. The human NEFL gene has been localized to chromosome 8p21 (62). Nfl, the murine homolog of human NEFL, has been mapped to mouse chromosome 14 and is within 3 cm of the Gnrh locus (GBASE). The close linkage of the mouse Raftk gene to Nfl (whose NEFL homolog is on human chromosome 8p21) suggested that the human RAFTK gene may be mapped to chromosome 8 based on homology between human and mouse chromosomes (62).

Therefore, we predict that the human RAFTK gene will be localized to chromosome 8p21. We have mapped the mouse Raftk gene to chromosome 14 using a C57BL/6J × M. spreitus F1 × M. spreitus backcross. The position of mouse Raftk was confirmed by RI line mapping using the BxD RI lines. The Raftk gene was also shown to be closely linked to Gnrh, whose human homolog (LHRH (luteinizing hormone-releasing hormone)) has been mapped to human chromosome 8p21:11.2 (42).

There has been considerable interest in the role of pp125FAK in signaling pathways in a variety of normal and transformed cells in response to different soluble and cell-surface stimuli. Future studies will aim to gain insights into the function of RAFTK in these signal transduction pathways, particularly distinguishing a role for RAFTK versus pp125FAK in these systems.

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