

Identification and Characterization of a Novel Tyrosine Kinase from Megakaryocytes*

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Protein-tyrosine kinases play pivotal roles in cell signal transduction. We have isolated a cDNA clone encoding a novel human intracytoplasmic tyrosine kinase, termed *matk* (megakaryocyte-associated tyrosine kinase). Expression of *matk* mRNA was predominantly found in cells of megakaryocytic lineage. The *matk* cDNA clone encodes a polypeptide of 527 amino acids and has closest sequence similarity to the *csk* tyrosine kinase. Sequence comparisons also indicate that *matk* contains *src* homology region 2 and 3 domains but lacks the NH₂-terminal myristylation signal, the negative regulatory tyrosine (Tyr-527), and the autophosphorylation site (Tyr-416) corresponding to those found in *src*. Antibodies raised against the NH₂ terminus of *matk* immunoprecipitated a 60-kDa protein from the CMK human megakaryocyte cell line. Expression of *matk* mRNA was up-regulated in megakaryocytic cells induced to differentiate by the phorbol ester. Based on its restriction in expression and its modulation during *in vitro* differentiation, it is likely that *matk* participates in signal transduction during megakaryocytopoiesis.

Protein-tyrosine kinases (PTKs)¹ play major roles in signal transduction pathways and include several oncoproteins and growth factor receptors (1, 2). The conservation of many tyrosine kinase sequences among organisms as divergent as invertebrates and mammals emphasizes the importance of these signaling molecules. Many PTKs span the cell membrane and function as receptor molecules for polypeptide ligands, while others are intracytoplasmic. Intracytoplasmic PTKs with homology to *src* have been described, which participate in signaling in normal and neoplastic blood cells (3–14). Although many intracytoplasmic PTKs are ubiquitously

expressed in a variety of cell types, several are relatively restricted in expression and mediate transduction of signals specific to cells of one lineage. Examples of restricted PTKs include *lck* (8) and *itk* (11), which mediate signal transduction in association with unique T cell-specific surface receptors and adhesion structures.

The most extensively characterized cytoplasmic PTKs belong to the *c-src* family. These intracellular PTKs are myristylated on glycine at position 2, which localizes them to the inner plasma membrane (15). Family members typically contain SH2 and SH3 domains, which are important for regulating enzyme activity. *src* family members also contain tyrosine residues at positions corresponding to amino acids 416 and 527 of *c-src*. Tyrosine 416 is an autophosphorylation site, and phosphorylation of tyrosine 527 results in attenuation of enzyme activity (16).

Csk, a PTK initially purified from rat brain (15) and subsequently cloned from rat, human, and chicken sources (17–19), does not appear to be a member of the *c-src* gene family (19). *Csk* lacks an autophosphorylation site within its kinase domain and is further distinguished from the *src* family of kinases by the lack of a carboxyl terminus equivalent of Tyr-527. *Csk* is the enzyme responsible for phosphorylation of Tyr-527 of *c-src* (16).

Relatively little is known about the repertoire of signal transduction molecules in human megakaryocytes (20, 21). Three transmembrane PTKs, the *c-Kit* protooncogene product and two fibroblast growth factor receptors (*blg* and *blk*), have been identified in human megakaryocytes (22–25). We have identified and characterized a novel intracytoplasmic kinase with some homology to *csk*, which is predominantly expressed in cells of megakaryocytic lineage. Given its homology to PTKs, we have termed this new gene megakaryocyte-associated tyrosine kinase (*matk*). Based on its molecular structure, its general restriction to cells of this lineage, and its increased expression during differentiation, it is likely that *matk* participates in signal transduction in human megakaryocytes.

MATERIALS AND METHODS

Cells—Human bone marrow was obtained by aspiration from the iliac crest of normal donors who gave informed consent in a protocol approved by the New England Deaconess Hospital Institutional Review Board. The marrow was aspirated into preservative-free heparin (Sigma) and separated by centrifugation through Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc.) at 1,200 × *g* at room temperature for 30 min. After two washes with sterile 1 × phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640 medium with 7.5% platelet poor plasma, penicillin/streptomycin (P/S), and L-glutamine, seeded onto T-75 tissue culture flasks (Corning, Corning, NY), and incubated at 37 °C in 5% CO₂. Human marrow megakaryocytes were isolated by a method employing immunomagnetic beads using anti-human glycoprotein GpIIb/IIIa monoclonal antibody as de-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L18974.

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¹ The abbreviations used are: PTK, protein-tyrosine kinase, SH, *src* homology; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

scribed previously (26). All of the isolated cells were recognizable as megakaryocytes by morphology and/or specific immunofluorescence using antiplatelet antibodies to the surface structures GpIIb/GpIIIa and GpIb. CD34 bearing marrow progenitor cells were purified from heparinized bone marrow aspirates using immunomagnetic beads coated with an anti-CD34 monoclonal antibody as described (27).

The CMK, CMK-6, and CMK 11-5 cell lines, provided by Dr. T. Sato and derived from a child with megakaryoblastic leukemia, have properties of cells of megakaryocytic lineage, including surface expression of glycoproteins Ib and IIb/IIIa, synthesis of platelet factor 4, platelet-derived growth factor, von Willebrand's factor, and become polyploid on induction with phorbol esters (28, 29). No myeloid or lymphoid surface markers have been found on our cultured CMK cells. The CMK cell lines were cultured in RPMI 1640 medium with 10% fetal calf serum.

Additional permanent human megakaryocytic cell lines studied were generous gifts to our laboratory. DAMI cells were from Dr. S. Greenberg, Brigham and Women's Hospital, Boston, MA, MEG-01 and Mo7E cells were from Dr. J. Hoxie, University of Pennsylvania, Philadelphia, and erythroid-megakaryocytic HEL cells and CHRF cells were from Dr. L. Zon, Children's Hospital, Boston, MA. Each cell line was cultured as previously described (30). All other permanent human cell lines were obtained from the American Type Tissue Culture Collection and maintained in liquid culture according to the specifications in the catalog.

In some experiments, megakaryocytic cells were induced to differentiate by treatment with phorbol 12-myristate 13-acetate (PMA) (Sigma). PMA was dissolved in dimethyl sulfoxide and stored at -20°C until use, when it was diluted in RPMI 1640 medium and used at 10 ng/ml.

DNA Amplification and Cloning—Total RNA was prepared by a standard protocol of lysis in guanidinium isothiocyanate followed by cesium chloride gradient centrifugation (31). Protein-tyrosine kinase sequences were amplified with degenerate oligonucleotide primers as described by Wilks (32).

PCR products of the amplified tyrosine kinase domains were purified from the agarose gel, digested with *EcoRI* and *BamHI*, ligated into pUC19, and transformed into *Escherichia coli* DH5 α . Sequencing was carried out by the dideoxy chain termination method using a sequenase kit (U. S. Biochemical Corp.). Sequences were compared with known sequences in GenBank and EMBL data bases using the Autosearch computer program to identify novel clones. The 160-base pair PCR product from clone number D4 was radiolabeled using the random priming protocol (31) and used as a probe to screen an oligo(dT)-primed cDNA library in λ gt11 prepared from CMK cells. Positive clones were isolated, plaque-purified, cDNA inserts excised, subcloned into Bluescript-SK (Stratagene), and sequenced on both complementary strands.

Northern Blot Analysis—Poly(A⁺) RNA was isolated directly from whole cells using an oligo(dT)-cellulose column kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. 2 μg was denatured and loaded onto a denaturing 1% agarose gel. Following electrophoresis, the RNA was vacuum blotted (Pharmacia LKB Biotechnology Inc.) onto a nylon filter (Nytran, Schleicher and Schuell) and baked at 80°C for 2 h. Hybridization was carried out according to the manufacturer's instructions. The filter was probed with a full-length *matk* cDNA radiolabeled to a high specific activity (10^6 – 10^9 cpm/ μg) with [α - ^{32}P]dCTP. After overnight incubation at 42°C , the blot was washed at high stringency conditions ($2 \times \text{SSC}$, 0.1% SDS twice for 15 min at room temperature, then $0.1 \times \text{SSC}$, 0.1% SDS once at 37°C , and once at 55°C) and then exposed to x-ray film at -70°C with an intensifying screen. The level of expression for each mRNA was determined densitometrically (EC Apparatus Corp. Densitometer; St. Petersburg, FL). The radioactivity associated with each band was assayed with a Betascope 603 blot analyzer (Betagen, Mountain View, CA). The ^{32}P -labeled probe was removed by incubating each RNA blot in 0.2 M EDTA, 0.05% pyrophosphate, 0.1 \times Denhardt's buffer, 5 mM Tris-HCl, pH 8.0, for 1 h at 65°C ; the same blot was assessed for the presence of the actin transcript. To determine the change in the level of a transcript, the radioactivity associated with *matk* mRNA was compared with the radioactivity associated with actin mRNA after correction for background.

The human tissue Northern blot was obtained from Clontech (Palo Alto, CA).

PCR Blots—cDNA was prepared from various cell lines and amplified by PCR (33) using specific *matk* primers. First-strand DNA was synthesized at 37°C for 1 h in a volume of 10 μl containing 4.5 μl of total RNA (4.5 μg) in diethyl pyrocarbonate- dH_2O , 50 mM Tris-

HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl_2 , and 50 $\mu\text{g}/\text{ml}$ actinomycin D, 20 units RNasin (Promega Corp.), 1.0 mM dNTPs (Pharmacia), 1 $\mu\text{g}/\mu\text{l}$ oligo(dT)16, and 0.2 units of Moloney murine leukemia virus reverse transcriptase (200 units/ml) (Boehringer Mannheim). The first-strand cDNA mix was combined with 90 μl of PCR mix that contained both *matk* primers at 1 μM , 0.2 mM dNTPs, 50 mM KCl, 0.1% gelatin, and 2.5 units of *Thermus aquaticus* thermostable DNA polymerase (Perkin-Elmer Cetus Instruments). The mixture was then subjected to PCR amplification using the Perkin-Elmer Cetus thermal cycler set for 40 cycles as follows: denature 94°C , 1 min; primer anneal 55°C , 2 min; primer extension 72°C , 3 min. 1-min ramp times were used between these temperatures. The sequence of the upstream primer was 5'-GCG GGG CGA GGC TCT CTG GTT-3 (position 265–285, Fig. 1). The nucleotide sequence of the downstream primer was 5'-TGC GAG CAC ACC CGC CCC AAG-3, (position 430–450, Fig. 1). The PCR products were electrophoresed on a 2% agarose gel, denatured, neutralized, transferred to filters, and vacuum blotted. The filters were baked at 80°C for 2 h and then prehybridized according to the manufacturer's instructions. The probe used was the full-length *matk* cDNA, which was labeled by random priming as described above. Hybridization was carried out as described previously (31) at 42°C in buffer containing 50% (v/v) formamide, and the blotted membrane was washed (31) at 62°C and then subjected to autoradiography.

In Vitro Transcription and Translation—2 μmol of template DNA (pBluescript-SK containing the entire coding region of *matk*) was linearized by digestion with *SaI*. Transcription was performed at 37°C for 1 h in a volume of 50 μl containing 10 mM dithiothreitol, 2.5 μg of bovine serum albumin, 0.25 mM each dNTP, 0.5 M m7GRNA cap (New England Biolabs, Beverly, MA), 2.5 units of RNasin (Promega Corp.), 3 units of T3 RNA polymerase (Pharmacia), and the template DNA. RNA was subsequently purified by addition of 1 μg of RNase-free DNase (Promega Corp.) for 15 min at 37°C then phenol/chloroform extraction and ethanol precipitation. Translation was performed using the rabbit reticulocyte lysate system (Promega Corp.) according to the manufacturer's instructions. [^{35}S]methionine (350 μCi) was used to label the translation product, which was then mixed with SDS sample buffer containing β -mercaptoethanol, boiled for 2 min, and electrophoresed on a 10% SDS-polyacrylamide gel. For the kinase assay, the *in vitro* translated products were added to 40 μl of kinase buffer (20 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 1 mM Na_3VO_4 , 4 μl of 0.03% enolase, and 5 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol). The mixtures were incubated for 25 min at room temperature and 5 min at 37°C . Products were fractionated by SDS/10% polyacrylamide gel electrophoresis, and detected by autoradiography for 16 h at -70°C with an intensifying screen.

Protein Analysis—Metabolic labeling, immunoprecipitation, and immunolocalization assays were performed in CMK cells as described previously (34–38). For immunoblot analysis, total lysates were prepared as described previously (34). Relative protein concentrations were determined with a colorimetric assay kit (Bio-Rad) with bovine serum albumin as the standard. A portion of lysate containing approximately 0.05 mg of protein was mixed with an equal volume of 2 \times SDS sample buffer containing 2-mercaptoethanol, boiled for 5 min, fractionated on 10% polyacrylamide-SDS gels (35) and transferred to Immobilon polyvinylidene difluoride (Millipore Corp., Bedford, MA) filters. Protein blots were treated with specific antipeptide antibodies (see below). Primary binding of the *matk*-specific antibodies was detected using anti-IgG second antibodies conjugated to horseradish peroxidase and subsequent chemiluminescence development ECL Western blotting system (Amersham International).

For metabolic labeling 10^6 cells were labeled with 100 μCi of [^{35}S]methionine in 1 ml of Dulbecco's modified Eagle's medium minus methionine (Amersham Corp.) for 16 h. Immunoprecipitation of *matk* protein from labeled cells with antipeptide antiserum was performed as described below (36). Portions of lysates containing 10^7 cpm of acid-insoluble [^{35}S]methionine were incubated with 1 μg of the antiserum in a 0.5 ml of reaction mixture. Immunoprecipitation samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

For immunolocalization studies, 10^7 CMK cells were resuspended in 1 ml of sonication buffer (60 mM Tris-HCl, pH 7.5, 6 mM EDTA, 15 mM EGTA, 0.75 M sucrose, 0.03% leupeptin, 12 mM phenylmethylsulfonyl fluoride, 30 mM 2-mercaptoethanol). Cells were sonicated 6 times for 10 s each and centrifuged at $25,000 \times g$ for 10 min at 4°C . The pellet was dissolved in 1 ml of sonication buffer and centrifuged at $25,000 \times g$ for 10 min at 4°C . The pellet (nucleus fraction) was resuspended in 1 ml of sonicated buffer and added to an equal volume

of 2 × SDS sample buffer. The supernatant obtained above (after the first sonication) was again centrifuged at 100,000 × *g* for 40 min at 4 °C. The supernatant (cytosolic fraction) was removed and added to an equal volume of 2 × concentrated SDS sample buffer. The remaining pellet (membrane fraction) was washed and dissolved in sonication buffer and SDS sample buffer as described above. Protein samples were analyzed by electrophoresis on 10% polyacrylamide gels, according to the Laemmli method (35). The proteins were transferred from the gels onto a 0.45-μm polyvinylidene difluoride membrane for subsequent immunoblot analysis. Primary binding of the *math*-specific antibodies was detected using anti-IgG second antibodies conjugated to horseradish peroxidase.

For immunohistochemical localization of *math* protein, CMK cells were grown on cover slips to approximately 50% confluency and were washed with PBS (pH 7.4) after removing the medium. The cells were prefixed for 1 min at 37 °C in 1% paraformaldehyde containing 0.075% Triton X-100, rinsed with PBS, and then fixed for 10 min with 4% paraformaldehyde. After the fixation step, cells were rinsed in PBS, quenched in PBS with 0.1 M glycine for 5 min, treated with PBS containing 0.1% Triton X-100, and finally rinsed again in PBS. For antibody staining, the cells were first blocked with a blocking solution (3% bovine serum albumin in PBS) and incubated for 1 h at 37 °C. The cells were then incubated for 1 h at 37 °C with *math* antiserum (1:100 dilution) or with preimmune rabbit serum (1:100)

(see below). After the incubation with the primary antibody, the cells were washed in PBS containing 3% bovine serum albumin and 0.1% Tween 20 and incubated for 1 h at 37 °C in fluorescein-conjugated donkey anti-rabbit IgGs (Jackson ImmunoResearch, Maine) diluted 1:1000 in blocking solution. The coverslips were washed in PBS and a drop of 1 mg/ml *p*-phenylenediamine in a mixture of PBS (pH 8.0), and glycerol was added to each coverslip before mounting on glass slides and sealing with clear nail polish. All glass slides were examined with a Zeiss Axiophot microscope.

Antibodies—The peptide SALD03 corresponding to amino acid residues 2–16 of the *math* protein was synthesized. Coupling of the peptide to carrier protein and immunizations was performed as described (39). Rabbit antibodies against this peptide were raised, and sera were titrated against peptide antigen by ELISA (40). The sera exhibiting the highest titer (1:27,000) were used in subsequent experiments.

RESULTS

Identification and Isolation of Full-length *math* cDNA—To identify tyrosine kinases in human megakaryocytes, we adapted the method of Wilks (32) that uses PCR primers based on conserved sequences of PTKs. RNA from the human megakaryocytic CMK cell line was used as a template to

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1  GGAGCAACTCGCTCCAAGTTGTGACGCCGGGACCGCTCGGGGTGTGCAGCCGGCTCGCGGAGGCCCTCTGGGGGCGGGCGGGCGCGGCTCGG
96  GGGCGCCCCCTGAGCAGAAAACAGGAAGAACAGGCTCGGTCCAGTGGCACCCAGCTCCCTACCTCCTGTGCCAGCCGCTGGCTGTGGCAGGC
191  CATTCCCAGCGTCCCCGACTGTGACCACTTGCTCAGTGTGCCTCTCACCTGCCTCAGTTCTCTTGGGGCGGGCGGGCGCGGCTCTGGT
10  20  30  40
SerTrpArgAlaPheHisGlyCysAspSerAlaGluGluLeuProArgValSerProArgPheLeuArgAlaTrpHisProProValSerAla
286  TTCTTGGCGGCATTTCACGGCTGTGATTCTGCTGAGGAACCTCCCGGGTGAGCCCCGCTTCCTCCGAGCTGGCACCCCCCTCCCGTCTCAG
50  60  70
ArgMetProThrArgArgTrpAlaProGlyThrGlnCysIleThrLysCysGluHisThrArgProLysProGlyGluLeuAlaPheArgLys
381  CCAGATGCCAACGAGGCGCTGGGCCCGGGCACCCAGTGTATCACCAATGCGAGCACACCGCCCCAAGCCAGGGAGCTGCGCTTCCGCAAG
80  90  100
GlyAspValValThrIleLeuGluAlaCysGluAsnLysSerTrpTyrArgValLysHisHisThrSerGlyGlnGluGlyLeuLeuAlaAlaGly
476  GCGGACGTGGTCACTATCCTGGAGGCTGCGAGAACAGAGCTGGTACCGCGTCAAGCACACACAGTGGACAGGAGGGGCTGCTGGCAGCTGG
110  120  130
AlaLeuArgAspGlyGluAlaLeuSerAlaAspProLysLeuSerLeuMetProTrpPheHisGlyLysIleSerGlyGlnGluAlaValGlnGln
571  GCGCTGCGGGACGGGAGGCGCTCTCCGACAGCCCAAGCTACGCTCATGCCGTGGTTCACGGGAAGATCTCGGGCCAGGAGGCTGTCCAGC
140  150  160
LeuGlnProProGluAspGlyLeuPheLeuValArgGluSerAlaArgHisProGlyAspTyrValLeuCysValSerPheGlyArgAspVal
666  AGCTGCAGCTCCCGAGGATGGGCTGTCTGGTGGCGGAGCTCCGCGGCCACCCCGCGACTACGTCTGTGCGTGAGCTTTGGCCGCGACGTC
170  180  190
IleHisTyrArgValLeuHisArgAspGlyHisLeuThrIleAspGluAlaValPhePheCysAsnLeuMetAspMetValGluHisTyrSerLys
761  ATCCACTACCGCTGTGTCACCGCGACGGCCACCTCAATCGATGAGGCGGTGTCTTCTGCAACCTCATGGACATGGTGGAGCATTACAGCAA
200  210  220
AspLysGlyAlaIleCysThrLysLeuValArgProLysArgLysHisGlyThrLysSerAlaGluGluGluLeuAlaArgAlaGlyTrpLeuLeu
856  GGACAAGGGCGCTATCTGCACCAAGCTGGTGAGACCAAGCGGAAACCGGACCAAGTCCGCCGAGGAGGAGCTGGCCAGGCGGGCTGGTTAC
240  250  260
AsnLeuGlnHisLeuThrLeuGlyAlaGlnIleGlyGluGlyGluPheGlyAlaValLeuGlnGlyGluTyrLeuGlyGlnLysValAlaVal
951  TGAACCTGCAGCATTTGACATTGGGAGCACAGATCGGAGAGGAGAGTTTGGAGCTCTCTGCAGGGTGAGTACCTGGGGCAAAGGTGGCCGTG
270  280  290
LysAsnIleLysCysAspValThrAlaGlnAlaPheLeuAspGluThrAlaValMetThrLysMetGlnHisGluAsnLeuValArgLeuLeuGly
1046  AAGAATATCAAGTGTGATGTGACAGCCAGGCTTCTTGGACGAGACGGCGCTCATGACGAGATGCAACACGAGAACCTGGTGCGTCTCTGGG
300  310  320
ValIleLeuHisGlnGlyLeuTyrIleValMetGluHisValSerLysGlyAsnLeuValAsnPheLeuArgThrArgGlyArgAlaLeuValAsn
1141  CGTGATCTGCACAGGGGCTGTACATTGTCTGAGCAGCTGAGCAAGGCAACCTGGTGAACCTTCTGCGGACCCGGGGTGCAGCCCTCGTGA
330  340  350
ThrAlaGlnLeuLeuGlnPheSerLeuHisValAlaGluGlyMetGluTyrLeuGluSerLysLysLeuValHisArgAspLeuAlaAlaArg
1236  ACACCGCTCAGCTCCTGCAGTTTTCTCTGCACGTGGCCGAGGGCATGGAGTACCTGGAGAGCAAGAAGCTTGTGCACCGCGACCTGGCCGCCGC
360  370  380
AsnIleLeuValSerGluAspLeuValAlaLysValSerAspPheGlyLeuAlaLysAlaGluArgLysGlyLeuAspSerSerArgLeuProVal
1331  AACATCTGGTCTCAGAGGACCTGGTGGCCAAGGTACGCACTTTGGCTGGCCAAAGCCGAGCGGAGGGCTAGACTCAAGCCGGCTGCCCGT
390  400  410
LysTrpThrAlaProGluAlaLeuLysHisGlyPheThrSerLysSerAspValTrpSerPheGlyValLeuLeuTrpGluValPheSerTyrGly
1426  CAAGTGGACGGCGCCGAGGCTCTCAACACGGGTTCAACAGCAAGTCCGATGTCTGGAGTTTGGGGTGCTGCTCGGGAGGTCTTCTCATATG
430  440  450
ArgGluProTyrProLysMetSerLeuLysGluValSerGluAlaValGluLysGlyTyrArgMetGluProProGluGlyCysProGlyPro
1521  GACGGCTCCGTACCTAAATGTCTACTGAAAGAGGTGTCCGAGGCGGTGGAGAGGGGTACCGCATGAACCCCCAGGGGTGTCAGGCCCC
460  470  480
ValHisValLeuMetSerSerCysTrpGluAlaGluProGluAlaGlyHisProSerAlaAsnTrpProArgSerTrpProGlySerTyrAlaVal
1616  GTGCACGTCTCATGAGCAGCTGCTGGGAGCGAGCGCCGCCGCCACCTTCCGCAAACTGGCCGAGAGCTGGCCCGGGAGCTACGCACT
490  500  510
GlnValProGlnProProSerGlnGlyArgThrProThrValHisLeuAlaProLysProGlyAlaLeuThrProProGlyGlyProTrpProGln
1711  GCAGGTGCCCCAGCTCCGTCTCAGGCGAGGACCGGACGCTCCACCTCGCCCCGAAGCCAGGAGCCCTGACCCACCCGGTGGCCCTTGGCCCC
520
ArgThrGluArgValGluSerAlaAlaTrpGlyHis
1806  AGAGGACCGAGAGAGTGGAGAGTGGCGGTGGGGGCACTGACAGGCCCAAGGAGGTCCAGGCGGCAAGTCATCTCTGGTGGCCACAGCAG
1901  GGCTGGCCACGTAGGGGGCTCTGGCGGCCCGTGACACCCAGACCTGCGAAGGATGATCGCCCGATAAAGACGGATTCTAAGG

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Fig. 1. Nucleotide and deduced amino acid sequence of two overlapping *math* cDNA clones representing the full-length cDNA. Nucleotide numbers are shown on left. The putative initiation codon at nucleotide position 263 is shown in bold type.

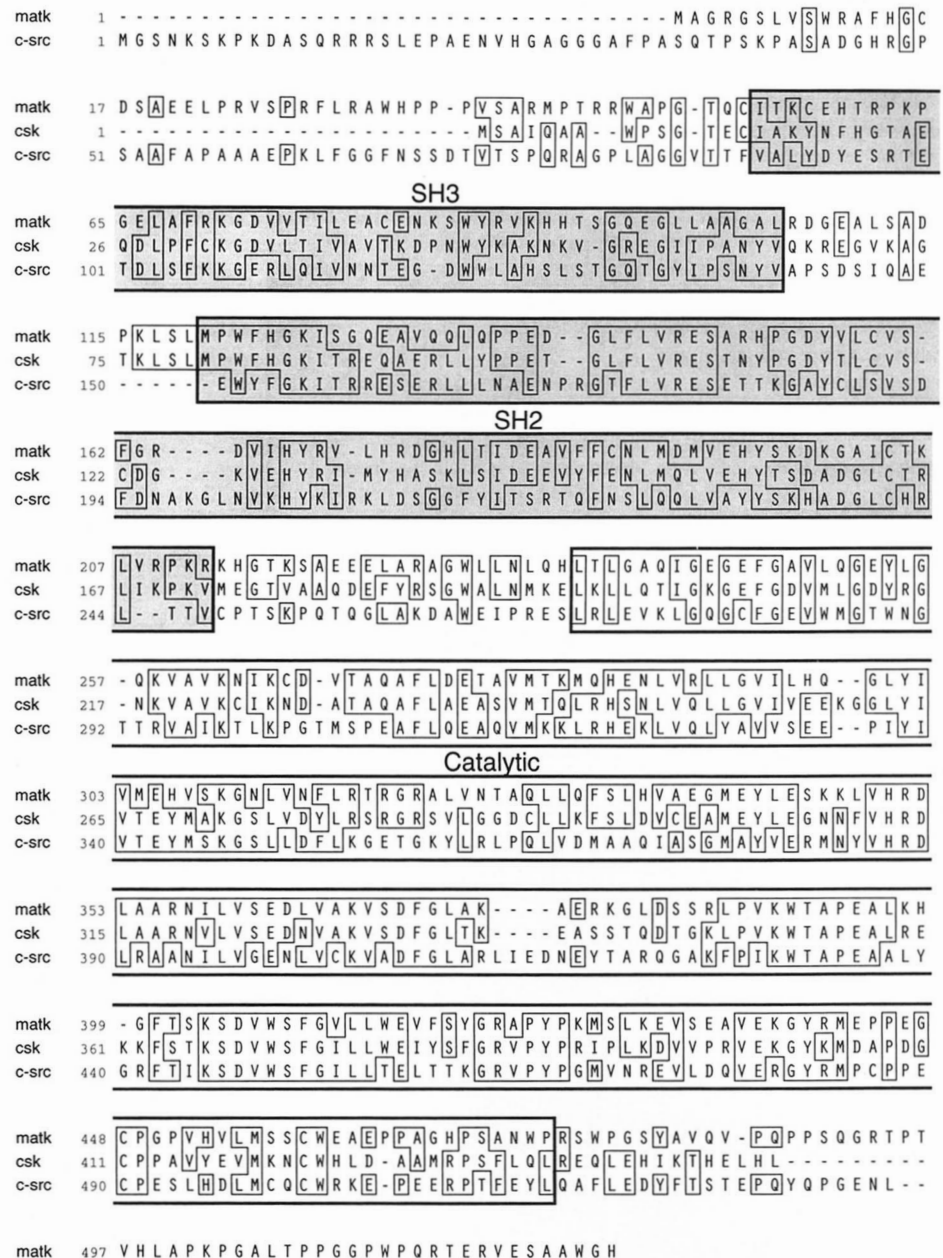


FIG. 2. Comparison of the deduced amino acid sequence of *matk* with those of human *csk* and human *c-src* proteins. Gaps (indicated by dashes) are introduced to optimize the alignment. Amino acid residues found to be conserved between *matk* and human *csk* or human *c-src* are boxed. The putative SH2 and SH3 domains are shaded, and the catalytic domain is boxed.

synthesize CMK cDNA. The cDNA was amplified by using the PTK primers. Fragments of the expected size (~160 base pairs) were isolated and subcloned for sequence analysis. The analysis of 190 independent clones identified several PTKs, including *c-kit*, *lyn*, *jitk-4*, *fms*, and *flt-4*. One clone that appeared to represent a novel tyrosine kinase was used as a probe to screen a CMK cDNA library. Two overlapping cDNA clones were isolated and sequenced. The length of the composite cDNA is 1987 base pairs (Fig. 1; accession no. L18974). The first methionine codon encountered at the 5' end of the cDNA (nucleotides 263–265) is preceded by an in-frame stop codon (position 212–214) and followed by a stop codon at position 1844–1846. The encoded polypeptide of 527 amino acids has a predicted molecular mass of 58,473 daltons and has been given the name *matk* (megakaryocyte-associated tyrosine kinase).

matk contains several structural motifs common to many *src*-related PTKs. A unique domain is found at the NH₂ terminus of *matk* (amino acids 1–50). This region is the most

divergent among various cytoplasmic PTKs and may be involved in cellular localization and/or interaction with other cellular proteins. SH3 and SH2 domains are found at amino acids 54–105 and 120–212, respectively (Fig. 2). These domains are thought to be important for regulating the enzymatic activity of intracellular PTKs, the SH3 domain through association with the cytoskeleton or membrane proteins (41), and the SH2 domain through its role as a phosphotyrosine binding site (42). The carboxyl part of *matk* (amino acid 235–527) consists primarily of the catalytic domain (amino acids 235–478), including the ATP binding site (amino acids 242–262). It also contains the highly conserved PTK sequence motifs HRDLAARN (HRDLRAAN) in *src* family proteins and PVKWTAPE (amino acids 350–357 and 387–394, respectively), which have been implicated in tyrosine phosphorylation specificity (43).

matk shares the highest degree of amino acid identity with the human *csk* (14) and *c-src* (44) gene products. The overall amino acid identity of *matk* with *csk* and *c-src* is 50 and 35%,

respectively (Fig. 2). The regions of highest homology are found in the catalytic domain (54% with *csk* and 44% with *c-src*) and the SH2 domain (55% with *csk* and 41% with *c-src*).

Although *matk* shares many common features with *src*, there are significant differences in other key motifs. Like *csk*, *matk* is missing a putative myristylation signal (glycine at position 2 and lysine or arginine at position 7), which is present in *src* family PTKs and is required for membrane localization (15). Similarly, *matk* lacks the autophosphorylation site corresponding to Tyr-416 of *src* as well as the negative regulatory tyrosine residue corresponding to *src* Tyr-527. These are conserved amino acids in most *src* family members but not in *matk* and *csk*. Taken together, these results indicate that *matk* is a member of the *csk* family.

Matk Is Highly Restricted in Tissue Expression—An extensive survey of permanent human cell lines and primary tissues was performed by Northern blot analysis. These experiments revealed that *matk* RNA (2.3 kilobases) is abundantly expressed in human megakaryocytic cell lines (Fig. 3A). No expression of *matk* was detected by Northern blot in primary tissues of various origins (heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas), with the exception of a 2.3-kilobase message in adult brain (Fig. 3B). Using PCR techniques, expression of *matk* was found in primary bone marrow megakaryocytes, blood platelets, and in marrow CD34⁺ progenitor cells (Table I). *matk* expression was also detected by PCR in the K562 pluripotent hematopoietic cell line, the primitive PLB leukemic line, and the MCF-7 breast cancer line but not by Northern blot. No expression of *matk* was seen in other hematopoietic cells including T cells, B cells, monocytes, or mast cells (Table I).

Regulation of *matk* Expression during PMA-induced Differentiation—To determine whether *matk* expression may be regulated during megakaryocytopoiesis, we have performed kinetic analysis of *matk* expression in CMK cells induced to differentiate *in vitro* by the phorbol ester PMA for 3, 6, or 24 h. This induction results in increased DNA content (polyploidy) and increased expression of surface platelet glycoprotein GpIb and GpIIb/GpIIIa. Northern blot analysis indicated 3–8-fold up-regulation of *matk* expression after 6 h of stimulation with PMA followed by down-regulation of *matk* expression after 24 h (Fig. 4A).

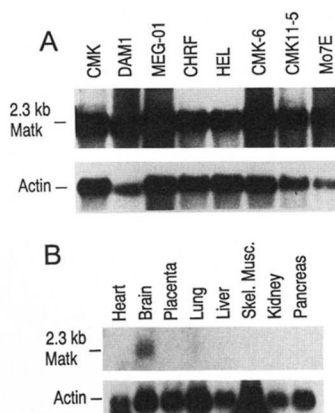


FIG. 3. *matk* expression. Panel A, expression of *matk* by Northern blot analysis in human megakaryocytic cell lines. Panel B, expression of *matk* by Northern blot analysis in human tissues. In both cases, poly(A⁺) RNA (2 μ g) was extracted from human cell lines or tissues, electrophoresed in a denatured 1% agarose-formaldehyde gel, and transferred to a nitrocellulose filter as described under "Materials and Methods." The filters were hybridized with ³²P-labeled *matk* cDNA, followed by hybridization with β -actin as the control for uniform RNA loading. *Skel. Musc.*, skeletal muscle.

TABLE I

matk expression by PCR in hematopoietic and nonhematopoietic cells

All samples were processed as described under "Materials and Methods." The PCR products were electrophoresed on a 2% agarose gel and hybridized with full-length *matk* cDNA as a probe. Expression was determined based on hybridization with *matk* cDNA. Plus (+) symbol indicates hybridization, and minus (–) symbol indicates no hybridization detected.

Cells	Expression
Bone marrow megakaryocytes	+
Platelets	+
Marrow CD34 ⁺ progenitor cells	+
K562, chronic myelogenous	+
PLB, premonocytic	+
U937, monocytic	–
THP-1, monocytic	–
KG-1, acute myelogenous	–
BL979, lymphoma	–
HG, T cell lymphoma	–
MoLT-4, T cells	–
KMT-2, CD34 ⁺ cells	–
RL-B cells	–
HT-Burkitt lymphoma	–
Ramos-Burkitt lymphoma	–
Raji-1-Burkitt lymphoma	–
MCF-7, breast adenocarcinoma	+
Hep 3B, liver carcinoma	–
B1, brain glioblastoma	–
U373, brain glioblastoma	–
H310, small cell lung carcinoma	–
HM-1, melanoma	–
EJ, bladder carcinoma	–
CoLo 205, colon carcinoma	–
PA-1, ovarian teratocarcinoma	–
Bone marrow fibroblasts	–
Human umbilical vein endothelial cells	–

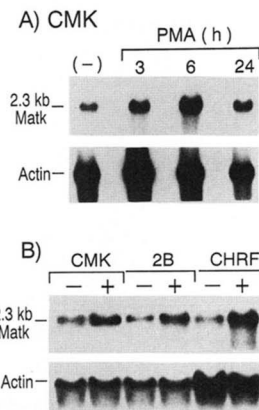


FIG. 4. Expression of *matk* in human megakaryocytic cell lines induced to differentiate by the phorbol diester. A, poly(A⁺) RNA (2 μ g), extracted from CMK cells with or without PMA treatment for 3, 6, and 24 h, was electrophoresed in a denatured 1% agarose-formaldehyde gel and transferred to nitrocellulose filters. Hybridization was performed with ³²P-labeled *matk* cDNA, followed by hybridization with ³²P-actin as the control. The exposure time used for all lanes with both probes was 10 h. B, poly(A⁺) RNA (2 μ g), extracted from human cell lines with or without PMA treatment for 6 h, was electrophoresed in a denatured 1% agarose-formaldehyde gel and transferred to nitrocellulose filters. The filters were hybridized with ³²P-labeled *matk* cDNA, followed by hybridization with β -actin as the control for uniform RNA loading. The *matk* transcript is 2.3 kilobases. The exposure time used was equivalent for all lanes with both probes for 6 h.

We next analyzed whether *matk* expression might be up-regulated during PMA-induced differentiation in the megakaryocytic cell lines 2B (a CMK subclone) and CHRF. Northern blot analysis (Fig. 4B) indicated up-regulation of *matk*

expression in CMK, 2B, and CHRF cells approximately 3–8-fold during 6 h of PMA induction (Fig. 4B). These results indicate up-regulation of *matk* expression during PMA-induced differentiation in megakaryocytic cells.

Detection of *matk* Protein, Kinase Activity, and Subcellular Localization—The expression of the *matk* gene product was investigated using an antiserum prepared in rabbits against the unique amino-terminal region (see “Materials and Methods”). The specificity of this antiserum was examined by immunoprecipitating *in vitro* translated *matk* protein labeled with [³⁵S]methionine (Fig. 5A). *In vitro* translated *matk* protein exhibiting a molecular mass of about 60 kDa was specifically precipitated using this antiserum. Following addition of [^γ-³²P]ATP to the *in vitro* translated product, phosphorylation was detected (Fig. 5B).

We subsequently used this rabbit antiserum for the detection of *matk* protein *in vivo* by immunoprecipitation. The CMK cell line was metabolically labeled with [³⁵S]methionine, and extracts were immunoprecipitated with anti-*matk* antiserum. A major protein species of 60 kDa was detected in CMK cells (Fig. 5C) as well as in other human megakaryocytic cell lines such as CMK-6 and Meg-01 (data not shown).

matk protein was localized in the cytoplasm of CMK cells by Western blot analysis of the nuclear, membrane, and cytoplasmic fractions (Fig. 5D). This localization of *matk* was confirmed by immunofluorescence analysis to be associated with the cytoplasm and not with the plasma membrane or nucleus (data not shown).

DISCUSSION

The phosphorylation of tyrosine residues in specific protein substrates by PTKs is a central cell signaling event modulating growth and differentiation. Characterization of lineage-restricted PTKs should therefore provide insights into unique

pathways of cell proliferation in normal and neoplastic states.

The technique of PCR using degenerate primers allowed us to identify in human megakaryocytic cells a novel intracytoplasmic tyrosine kinase, which we have termed *matk*. *matk* is expressed predominantly in the megakaryocyte cell lineage. Sequence analysis of *matk* demonstrates homology to *csk*, suggesting that *matk* belongs to this subfamily of cytoplasmic tyrosine kinases. The features of the *csk* subfamily include the lack of myristylation signals, the lack of carboxyl-terminal regulatory phosphorylation site, and the presence of SH2 and SH3 domains. The SH2 domain (amino acids 121–207 in *matk*) is believed to interact with phosphotyrosines in protein substrates (2, 45, 46) and may modulate the enzymatic activity of *src* proteins. The SH3 domain (amino acids 54–105 in *matk*) can bind to proline-rich domains, and it may interact with the cytoskeleton (2, 38, 47). The putative amino terminus of *matk* shares limited homology with other intracytoplasmic PTKs, including those of the *csk* family. This region of the protein could be important in unique signal functions of *matk* or its association with specific cell structures in the megakaryocyte.

matk protein is located in the cytoplasm of megakaryocytic cells as determined by immunoblot analysis of subcellular fractions and by immunofluorescence staining. This result is consistent with the predicted lack of a myristylation signal. Several PTKs that are located in the cytosolic fraction have been reported including *csk* (17), *c-fps* (48), and FAK (49).

The phosphorylation of Tyr-527 of *c-src* by *csk* results in attenuation of *c-src* kinase activity (16). Similarly, *csk* phosphorylates the equivalents of Tyr-527 in other *src*-family PTKs, with resultant down-regulation of their activities *in vitro*. This phosphorylation of *c-src* has not yet been demonstrated *in vivo*. In yeast, phosphorylation of Tyr-527 of co-expressed *c-src* by *csk* eliminated *c-src*-mediated growth inhibition (46). It will thus be of interest to elucidate the role of *matk* in the phosphorylation of *src* family tyrosine kinases in megakaryocytes. Demonstration of substrate specificity similar to *csk* will be pursued in future studies using purified *matk* protein.

Expression of the *matk* gene appears highly restricted, with abundant expression observed by Northern blot only in cells of the megakaryocyte lineage. An extensive tissue survey demonstrates expression of *matk* in brain adult tissue. Within the hematopoietic system, expression of *matk* was uniform and high in megakaryocytic cells with low level expression detected only by PCR in the cell lines K562 and PLB and in marrow CD34 bearing progenitor cells. Because megakaryocyte precursors bear the CD34 surface structure (50), further work is required to determine if *matk* is present in progenitor cells committed to this lineage or in multipotential hematopoietic progenitors.

The homology to other intracytoplasmic PTKs and the restriction in tissue expression suggest that *matk* may function in signal transduction pathways important in megakaryocyte growth and/or differentiation. Our initial examination of *matk* expression during PMA treatment of a number of megakaryocyte cell lines revealed its up-regulation during cellular differentiation. Future studies will aim to better understand the role of *matk* in megakaryocyte signal transduction, particularly that mediated by cytokines and adhesive interactions that may modulate megakaryocytopoiesis.

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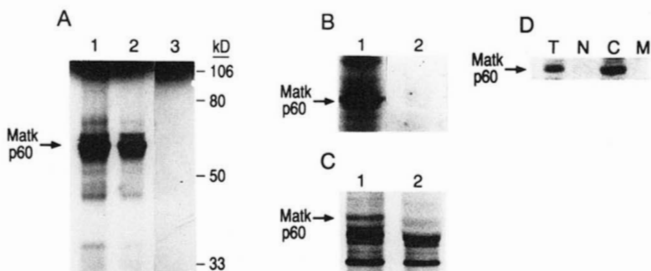


FIG. 5. The *matk* protein and its kinase activity. Panel A, *matk* cDNA cloned into pBluescript was transcribed *in vitro* under the control of the T7 promoter. Transcribed RNA was translated in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine (350 μ Ci) (lane 1). The *in vitro* translated product was immunoprecipitated by an anti-*matk* antiserum (lane 2) or preimmune serum (lane 3). Analysis was by SDS-polyacrylamide gel electrophoresis as described under “Materials and Methods.” The positions of molecular mass markers are indicated at right. Panel B, kinase activity of *matk* protein *in vitro*. The *in vitro* translated product was assayed for kinase activity by adding [^γ-³²P]ATP in 20 mM PIPES (pH 7.0) containing 20 mM MgCl₂ at 30 °C for 15 min (lane 1). Brown mosaic virus RNA was *in vitro* translated and assayed for kinase activity under identical conditions to serve as a negative control (lane 2). Products were analyzed by SDS-polyacrylamide gel electrophoresis. Panel C, detection of *matk* protein *in vivo*. CMK cells were metabolically labeled with 100 μ Ci of [³⁵S]methionine for 16 h, and the cell lysate was immunoprecipitated by anti-*matk* antiserum (1:100) (lane 1) or by preimmune serum (1:100) (lane 2). Panel D, immunolocalization of *matk* p60 protein by Western blot in CMK cells. By using anti-*matk* antiserum (dilution of 1:100), fractions were prepared and analyzed as described under “Materials and Methods.” Lane designations are as follows: T, total lysate; N, nuclear fraction; C, cytosol fraction; M, membrane fraction.

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