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Prokaryotic Expression Cloning of a Novel Human Tyrosine Kinase

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Screening of a human embryonic lung fibroblast cDNA expression library with antiphosphotyrosine antibodies led to isolation of a novel protein kinase. A clone, designated A6, contained a 3-kb cDNA insert with a predicted open reading frame of 350 amino acids. DNA sequence analysis failed to reveal any detectable similarity with previously known genes, and the predicted A6 protein lacked any of the motifs commonly conserved in the catalytic domains of protein kinases. However, the bacterially expressed β-galactosidase–A6 fusion protein demonstrated both tyrosine and serine phosphorylation in an in vitro kinase assay and phosphorylated exogenous substrates including myelin basic protein specifically on tyrosine residues. The enzyme also displayed biochemical properties analogous to those of other protein tyrosine kinases. The A6 gene was found to be expressed widely at the transcript level in normal tissues and was evolutionarily conserved. Thus, A6 represents a novel tyrosine kinase which is highly divergent from previously described members of this important class of regulatory molecules.

Protein phosphorylation has been implicated in a number of physiological processes ranging from normal cell growth and differentiation to malignancy. Particular interest has focused on the role of tyrosine phosphorylation in signal transduction pathways. This posttranslational modification is the primary signaling mechanism of many growth factor receptors and a number of oncogene products (1, 10). Changes in tyrosine phosphorylation are thought to be responsible for initiating a cascade of events leading to the biochemical modification of proteins, including other kinases, that regulate entry into and progression through the cell cycle.

To elucidate the mechanisms involved in the control of proliferation, efforts have focused on identifying novel members of the protein kinase family. Two major approaches have been used in this pursuit. The first involves low-stringency molecular hybridization and PCR techniques to detect sequences related to known genes (15, 27), whereas the second takes advantages of expression cloning since it does not depend on prior knowledge of sequence information (20). Furthermore, the latter technique, when applied to bacteria, is very useful for identifying protein tyrosine kinases since bacteria lack these enzymes. The success of expression-cloning strategies has been illustrated by the use of antiphosphotyrosine (anti-pTyr) antibodies for the identification and characterization of several novel kinases isolated from bacterial expression systems (13, 18).

Most protein kinases have been classified into two categories according to their primary structure and their specificity with respect to phosphorylation of either tyrosine or serine and threonine residues (7). However, several kinases recently identified with anti-pTyr antibodies, although having structures predictive of serine/threonine kinases, have been shown to phosphorylate tyrosine as well as serine and threonine residues when examined in bacterial expression systems or in vitro assays (3, 5, 22, 26). This has led to the classification of a third group of enzymes referred to as dual-specificity protein kinases (19).

In the present report, we describe the prokaryotic expression cloning and in vitro characterization of a novel protein kinase from a human embryonic lung fibroblast cDNA library by using an anti-pTyr monoclonal antibody. When expressed as a β-galactosidase fusion protein, this molecule demonstrates the ability to phosphorylate tyrosine as well as serine residues. Sequence analysis revealed that the predicted protein lacks any of the consensus motifs commonly found in protein kinases, although its in vitro biochemical properties resemble those of previously known members of the protein kinase family.

MATERIALS AND METHODS

Library construction and screening. The vector for prokaryotic expression, λpCEVlacz, was designed as previously described (12). An expression library was constructed by using oligo(dT)-primed M426 human lung fibroblast cDNA packaged into λpCEVlacz. For library screening, *Escherichia coli* Y1090 was infected with phage (2×10^4 plaques per 150-mm plate) and plated on agar plates containing ampicillin (50 μg/ml). After a 4-h incubation at 37°C, the plates were overlaid with nitrocellulose filter pretreated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 6 h at 37°C. Filters were rinsed in TTBS (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) and then blocked with 3% nonfat dried milk in TTBS for 60 min at room temperature. Positives clones were detected by probing filters with a mouse anti-pTyr monoclonal antibody (Upstate Biotechnology, Inc.) overnight, washing them twice with TTBS, and performing a secondary screening with ^{125}I-protein A for 60 min. The filters were washed four times with TTBS, dried, and exposed to Kodak XAR film overnight at −70°C. Plaques giving positive signals

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were isolated and subjected to secondary and tertiary screenings to ensure plaque purification.

Nucleotide sequence analysis. The cDNA inserts from plaque-purified clones were sequenced by the dideoxy-chain termination method (24) with T7 polymerase (U.S. Biochemical). Although λpCEVlacz is a phagemid vector allowing for efficient plasmid rescue, the existence of internal *Not*I and *Xho*I sites in clone A6 prohibited rescue of the full-length cDNA insert. Therefore, the A6 cDNA insert was excised with *Sal*I and subcloned into the *Sal*I sites of plasmid pUC18 (Boehringer Mannheim) for sequence determination.

In vitro transcription and translation. Plasmids designated pCEV27-A6 were analyzed for in vitro transcription/translation by using the TNT Coupled Reticulocyte Lysate System (Promega). Purified plasmids (1 μg) were added to a rabbit reticulocyte lysate in the presence of SP6 RNA polymerase and 40 μCi of [^{35}S]methionine (10 mCi/ml; specific activity, 1,078 Ci/mmol; translation grade; Dupont, NEN) as specified by the manufacturer (Promega). A 5-μl volume of sample was mixed with 20 μl of 2× sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Gels were dried and exposed to X-ray film at −70°C for 5 to 12 h.

Bacterial expression of β-gal–A6. *Escherichia coli* Y1089 cells were grown to an optical density at 600 nm of 0.4 in NZY medium and infected with either λpCEVlacz or λpCEVlacz-A6 at a multiplicity of infection of 10:1. The cells were then grown in the presence of 100 μM IPTG for 2 h at 37°C. For analysis of bacterial lysates, 1-ml aliquots were pelleted and resuspended in 100 μl of 2× SDS sample buffer. Then 20 to 40 μl was loaded onto SDS-polyacrylamide gels for analysis. For immunoprecipitations, cells were pelleted, resuspended in Nonidet P-40 lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 100 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin per ml), and lysed by sonication. Lysates were clarified by centrifugation at 14,000 × *g* for 10 min.

Western immunoblotting of bacterial lysates. Bacterial lysates were resolved on an SDS–7.5% polyacrylamide gel and transferred to Immobilon-P. Membranes were blocked with 3% nonfat dried milk in TTBS and then probed with either mouse monoclonal anti-β-galactosidase (β-gal) (Promega) or anti-pTyr antibody for 12 to 18 h at room temperature. Blots were washed twice with TTBS and then incubated with a 1:1,000 dilution of ^{125}I-protein A for 60 min. They were then washed four times with TTBS and exposed to X-ray film at −70°C.

In vitro kinase assay. Cleared lysates were immunoprecipitated with either anti-β-gal or anti-pTyr antibody and washed twice with 1 ml of Nonidet P-40 lysis buffer and twice with 1 ml of kinase reaction buffer (50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.4], 1% Nonidet P-40). Immunoprecipitates were resuspended in kinase reaction buffer containing 10 mM $MnCl_2$ and assayed for kinase activity by addition of 20 μCi of [γ-^{32}P]ATP to immunocomplexes for 20 min at room temperature. Myelin basic protein and poly(Glu-Tyr) 4:1 (Sigma) were included as exogenous substrates when indicated. Reactions were terminated by the addition of 2× SDS sample buffer, and ^{32}P-labeled proteins were resolved on SDS-polyacrylamide gels. In some cases proteins were transferred to Immobilon-P membranes. ^{32}P-labeled bands were visualized by autoradiography after exposure of dried gels or Immobilon-P membranes to X-ray film at −70°C.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed as previously described (8). Briefly, bands of interest were hydrolyzed from Immobilon-P membranes by incubation with 6 M HCl (constant-boiling grade; Pierce) for 2 h at 110°C. Samples volumes were adjusted to 1 ml with water, and HCl was removed under vacuum. Hydrolysates were washed with water; centrifuged under vacuum; resuspended in a mixture of phosphoserine, phosphothreonine, and pTyr (1 mg/ml each); and subjected to thin-layer electrophoresis. Two-dimensional analysis was performed at pH 1.9 for 60 min at 1 kV (first dimension) and at pH 3.5 for 50 min at 1 kV (second dimension); one-dimensional analysis was performed at pH 3.5 for 60 min. Phosphorylated amino acid standards were revealed by ninhydrin staining.

RNA preparation and Northern (RNA) analysis. M426 monolayers, grown in 100-mm tissue culture dishes, were washed in sterile phosphate-buffered saline, lysed in the presence of RNAzol (TelTest Inc.), and extracted with chloroform. Human tissues were pulverized in liquid nitrogen and homogenized with a polytron. RNA was recovered by precipitation with cold isopropanol. Typically, 20 μg of RNA was electrophoresed on 1% formaldehyde agarose gels and transferred to Nytran nylon membranes. After crosslinking of the RNA to the membrane, filters were prehybridized for 2 h at 42°C in Hybrisol (Oncor, Gaithersburg, Md.) (50% formamide, 10% dextran sulfate, 1% SDS, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], blocking agents) and then hybridized for 20 h in the same solution containing a ^{32}P-labeled probe corresponding to the 3-kb *Sal*I fragment of the A6 cDNA. Filters were washed twice (for 30 min each) at room temperature in 2× SSC–1% SDS and twice at 50°C in 0.1× SSC–1% SDS and exposed to Kodak XAR film.

Nucleotide sequence accession number. The GenBank accession number of the A6 cDNA sequence is U02680.

RESULTS

Expression cloning of a novel tyrosine protein kinase. An M426 human embryonic lung fibroblast bacterial expression library was screened with anti-pTyr antibody. Previous reports have demonstrated that bacterial cells lack tyrosine phosphorylation and that detection of phosphotyrosine in bacteria is dependent on the expression of recombinant protein kinases (18, 20). The majority of pTyr-immunoreactive clones detected in this screen were identified by sequence analysis as the tyrosine protein kinase gene *src* or *fyn* (data not shown). However, one clone, designated A6, contained a 3-kb insert possessing little similarity in its predicted sequence to any known proteins. To confirm the kinase function of A6, we characterized its expression in bacterial cells. As shown in Fig. 1A, SDS-PAGE analysis of lysates of cells infected with λpCEVlacz-A6 demonstrated IPTG-induced expression of a 150-kDa protein. In contrast, the λpCEVlacz expression vector alone encoded only the 110-kDa β-gal protein (Fig. 1A). Thus, the deduced molecular mass of the A6 expression product itself was approximately 40 kDa. To confirm that the 150-kDa protein was a β-gal fusion product, we analyzed bacterial lysates by immunoblotting analysis with anti-β-gal antibody. As shown in Fig. 1B, the λpCEVlacz-A6 product was detected as a predominant immunoreactive species of approximately 150 kDa, consistent with the size of the IPTG-inducible protein.

To characterize the protein kinase activity of the β-gal–A6 fusion protein, lysates from bacteria expressing β-gal–A6 or β-gal alone were analyzed by immunoblotting with anti-pTyr antibody. A predominant 150-kDa species was detected in β-gal–A6 lysates, consistent with the signal of the β-gal–A6

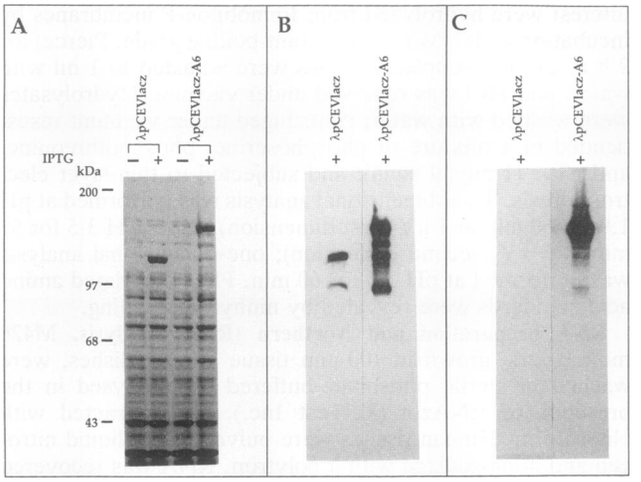

FIG. 1. Bacterial expression of β-gal–A6. *E. coli* 1089 was infected with either λpCEVlacz or λpCEVlacz-A6, induced with 100 μM IPTG for 2 h at 37°C, and lysed in 2× SDS sample buffer. Total bacterial lysates were separated by SDS-PAGE (7.5% polyacrylamide) (A) Coomassie stain of bacterial lysates from uninduced (lanes −) or induced (lanes +) infected bacteria. Lysates separated by electrophoresis were transferred to an Immobilon-P membrane. (B and C) Filters were immunoblotted with either anti-β-gal (panel B) or anti-pTyr (panel C) antibody. Positions of molecular mass standards are indicated.

fusion protein (Fig. 1C). Of note, a number of additional anti-pTyr antibody immunoreactive species were detected in the β-gal–A6 lysates. Although lower-molecular-mass species could represent breakdown products of β-gal–A6, pTyr immunoreactive species greater than 150 kDa were also observed. These findings suggested that expression of the β-gal–A6 fusion protein was associated with tyrosine phosphorylation of endogenous bacterial proteins as well. The immunoreactivity of anti-pTyr antibody with the β-gal–A6 product, as well as with other anti-pTyr antibody detectable species, was completely blocked by pretreatment of lysates with alkaline phosphatase or by preadsorbtion of antibody with 1 mM pTyr but not 1 mM phosphoserine or phosphothreonine (data not shown). To rule out potential artifacts arising from its expression as a β-gal fusion protein, A6 was also expressed as a polyhistidine-tagged protein and shown to be reactive with anti-pTyr antibody (data not shown). All of these results strongly implied that β-gal–A6 was tyrosine phosphorylated in bacteria.

Nucleotide and predicted amino acid sequence of A6. Figure 2 shows the nucleotide and deduced amino acid sequence of the A6 cDNA. The open reading frame encompassed 1,050 bp beginning with a methionine codon at position 61 and ending with a TAA termination codon at nucleotide 1110. The open reading frame was flanked by 5′ and 3′ untranslated regions consisting of 60 and 1890 nucleotides, respectively, with a potential polyadenylation signal (AATAAA) at position 2953. Assignment of codon 61 as the initiation codon is in agreement with the consensus initiation sequence established by Kozak (14). However, the absence of in-frame stop codons prior to nucleotide 61 precluded definitive assignment. Translation of the open reading frame starting at codon 61 predicted a 350-amino-acid polypeptide with a calculated molecular mass of 40,285 Da, consistent with the size of the β-gal–A6 fusion product (Fig. 1A).

To search for clones that contained additional 5′ sequence, as well as to examine the translational product of the

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                                                                  M  S  H  Q  T  G  I  Q  A  S  E  D  V
   1  CCGCCGGCCGGGGCGCCTGGCTGCACTCAGCGCCGGAGCCGGGAGCTAGCGGCCGCCGCCATGTCCCACCAGACCGGCATCCAAGCAAGTGAAGATGTTA
  14  K  E  I  F  A  R  A  R  N  G  K  Y  R  L  L  K  I  S  I  E  N  E  Q  L  V  I  G  S  Y  S  Q  P  S  D
 101  AAGAGATCTTTGCCAGAGCCAGAAATGGAAAGTACAGACTTCTGAAAATATCTATTGAAAATGAGCAACTTGTGATTGGATCATATAGTCAGCCTTCAGA
  47  S  W  D  K  D  Y  D  S  F  V  L  P  L  L  E  D  K  Q  P  C  Y  I  L  F  R  L  D  S  Q  N  A  Q  G
 201  TTCCTGGGATAAGGATTATGATTCCTTTGTTTTACCCCTGTTGGAGGACAAACAACCATGCTATATATTATTCAGGTTAGATTCTCAGAATGCCCAGGGA
  81  Y  E  W  I  F  I  A  W  S  P  D  H  S  H  V  R  Q  K  M  L  Y  A  A  T  R  A  T  L  K  K  E  F  G
 301  TATGAATGGATATTCATTGCATGGTCTCCAGATCATTCTCATGTTCGTCAAAAAATGTTGTATGCAGCAACAAGAGCAACTCTGAAGAAGGAATTTGGAG
 114  G  G  H  I  K  D  E  V  F  G  T  V  K  E  D  V  S  L  H  G  Y  K  K  Y  L  L  S  Q  S  S  P  A  P  L
 401  GTGGCCACATTAAAGATGAAGTATTTGGAACAGTAAAGGAAGATGTATCATTACATGGATATAAAAAATACTTGCTGTCACAATCTTCCCCTGCCCCACT
 147  T  A  A  E  E  E  L  R  Q  I  K  I  N  E  V  Q  T  D  V  G  V  D  T  K  H  Q  T  L  Q  G  V  A  F
 501  GACTGCAGCTGAGGAAGAACTACGACAGATTAAAATCAATGAGGTACAGACTGACGTGGGTGTGGACACTAAGCATCAAACACTACAAGGAGTAGCATTT
 181  P  I  S  R  E  A  F  Q  A  L  E  K  L  N  N  R  Q  L  N  Y  V  Q  L  E  I  D  I  K  N  E  I  I  I
 601  CCCATTTCTCGAGAAGCCTTTCAGGCTTTGGAAAAATTGAATAATAGACAGCTCAACTATGTGCAGTTGGAAATAGATATAAAAAATGAAATTATAATTT
 214  L  A  N  T  T  N  T  E  L  K  D  L  P  K  R  I  P  K  D  S  A  R  Y  H  F  F  L  Y  K  H  S  H  E  G
 701  TGGCCAACACAACAAATACAGAACTGAAAGATTTGCCAAAGAGGATTCCCAAGGATTCAGCTCGTTACCATTTCTTTCTGTATAAACATTCCCATGAAGG
 247  D  Y  L  E  S  I  V  F  I  Y  S  M  P  G  Y  T  C  S  I  R  E  R  M  L  Y  S  S  C  K  S  R  L  L
 801  AGACTATTTAGAGTCCATAGTTTTTATTTATTCAATGCCTGGATACACATGCAGTATAAGAGAGCGGATGCTGTATTCTAGCTGCAAGAGCCGTCTGCTA
 281  E  I  V  E  R  Q  L  Q  M  D  V  I  R  K  I  E  I  D  N  G  D  E  L  T  A  D  F  L  Y  E  E  V  H
 901  GAAATTGTAGAAAGACAACTACAAATGGATGTAATTAGAAAGATCGAGATAGACAATGGGGATGAGTTGACTGCAGACTTCCTTTATGAAGAAGTACATC
 314  P  K  Q  H  A  H  K  Q  S  F  A  K  P  K  G  P  A  G  K  R  G  I  R  R  L  I  R  G  P  A  E  T  E  A
1001  CCAAGCAGCATGCACACAAGCAAAGTTTTGCAAAACCAAAAGGTCCTGCAGGAAAAAGAGGAATTCGAAGACTAATTAGGGGCCCAGCGGAAACTGAAGC
 347  T  T  D  *
1101  TACTACTGATTAAAGTCATCACATTAAACATTGTAATACTAGTTTTTTAAAAGTCCAGCTTTTAGTACAGGAGAACTGAAATCATTCCATGTTGATATAA
1201  AGTAGGGAAAAAAATTGTACTTTTTGGAAAATAGCACTTTTCACTTCTGTGTGTTTTTAAAATTAATGTTATAGAAGACTCATGATTTCTATTTTTGAGT
1301  TAAAGCTAGAAAAGGGTTCAACATAATGTTTAATTTTGTCACACTGTTTTCATAGCGTTGATTCCACACTTCAAATACTTCTTAAAATTTTATACAGTTG
1401  GGCCAGTTCTAGAAAGTCTGATGTCTCAAAGGGTAAACTTACTACTTTCTTGTGGGACAGAAAGACCTTAAAATATTCATATTACTTAATGAATATGTTA
1501  AGGACCAGGCTAGAGTATTTTCTAAGCTGGAAACTTAGTGTGCCTTGGAAAAGCCGCAAGTTGCTTACTCCGAGTAGCTGTGCTAGCTCTGTCAGACTGT
1601  AGGATCATGTCTGCAACTTTTAGAAATAGTGCTTTATATTGCAGCAGTCTTTTATATTTGACTTTTTTTTAATAGCATTAAAATTGCAGATCAGGCTCACT
1701  CTGAAACTTTAAGGGTACCAGATATTTTCTATACTGCAGGATTTCTGATGACATTGAAAGACTTTAAACAGCCTTAGTAAATTATCTTTCTAATGCTCTG
1801  TGAGGCCAAACATTTATGTTCAGATTGAAATTTAAATTAATATCATTCAAAAGGAAACAAAAAATGTTGAGTTTTAAAAATCAGGATTGACTTTTTTCTC
1901  CAAAACCATACATTTATGGGCAAATTGTGTTCTTTATCACTTCCGAGCAAATACTCAGATTTAAAATTACTTTAAAGTCCTGGTACTTAACAGGCTAACG
2001  TAGATAAACACCTTAATAATCTCAGTTAATACTGTATTTCAAAACACATTTAACTGTTTTCTAATGCTTTGCATTATCAGTTACAACCTAGAGAGATTTT
2101  GAGCCTCATATTTCTTTGATACTTGAAATAGAGGGAGCTAGAACACTTAATGTTTAATCTGTTAAACCTGCTGCAAGAGCCATAACTTTGAGGCATTTTC
2201  TAAATGAACTGTGGGGATCCAGGATTTGTAATTTCTTGATCTAAACTTTATGCTGCATAAATCACTTATCGGAAATGCACATTTCATAGTGTGAAGCACT
2301  CATTTCTAAACCTTATTATCTAAGGTAATATATGCACCTTTCAGAAATTTGTGTTCGAGTAAGTAAAGCATATTAGAATAATTGTGGGTTGACAGATTTT
2401  TAAAATAGAATTTAGAGTATTTGGGGTTTTGTTTGTTTACAAATAATCAGACTATAATATTTAAACATGCAAAATAACTGACAATAATGTTGCACTTGTT
2501  TACTAAAGATATAAGTTGTTCCATGGGTGTACACGTAGACAGACACATACACCCAAATTATTGCATTAAGAATCCTGGAGCAGACCATAGCTGAAGCT
2601  GTTATTTTCAGTCAGGAAGACTACCTGTCATGAAGGTATAAAATAATTTAGAAGTGAATGTTTTTCTGTACCATCTATTGCAAATTATACTCTAAATTCC
2701  ACTACACTACATTAAAGTAAATGGAGATTCCAGAATATAGATGTGATTATAGTCTTAAACTAATTATTATTAACCAATGATTGCTGAAAATCAGTGATG
2801  CATTTGTTATAGAGTATAACTCATCGTTTACAGTATGTTTAGTTGGCAGTATCATACCTAGATGGTGAATAACATATTCCCAGTAAATTTATATAGCAG
2901  TGAAGAATTACATGCCTTCTGGTGGACATTTTATAAGTGCATTTTATATCACAATAAAAATTTTTTCTCTTTAAAAAAAAAAAACAAGAAAAAAAAAAA
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FIG. 2. Nucleotide sequence and predicted amino acid sequence of the A6 cDNA. Nucleotides and amino acids are numbered on the left, and single-letter amino acid designations are used. The figure was prepared with the DNA draw program of M. Shapiro, National Institutes of Health.

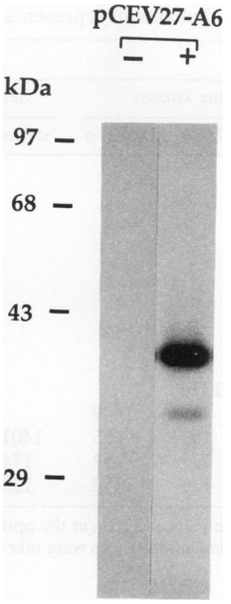

FIG. 3. In vitro translation of A6 cDNA. Plasmids designated pCEV27-A6 were subjected to in vitro transcription and translation as described in Materials and Methods. Reaction mixtures from rabbit reticulocyte lysate incubated alone (left lane) or in the presence of pCEV27-A6 (right lane) were analyzed by SDS-PAGE (10% polyacrylamide). The gel was dried and exposed to X-ray film for 8 h. Position of molecular mass standards are indicated.

cDNA, we used the 3-kb fragment as a probe to screen an oligo(dT)-primed cDNA library prepared from M426 cells. Analysis of an additional nine positive clones revealed that none encoded more 5′ sequence than previously obtained. When subjected to in vitro transcription/translation analysis, the major protein species detected had a relative molecular mass of ~40 kDa, consistent with the size predicted from the A6 coding sequence (Fig. 3).

Computer analysis with the Prosite program revealed that the predicted sequence lacked an amino-terminal secretory signal sequence or any other hydrophobic stretch characteristic of a membrane-spanning domain. A consensus N-myristoylation site was found at Gly-6. Several potential protein kinase C phosphorylation sites were identified at Thr-107, Thr-124, Ser-233, Ser-265, and Ser-274. Two consensus tyrosine kinase phosphorylation sites were also found at Tyr-236 and Tyr-249. To search for similarity between A6 and other previously identified proteins, we analyzed data bases by using the National Center for Biotechnology Information (NCBI) BLAST network service (2). Searches of NCBI data bases (SWISS-PROT 25.0, PIR 36.0, and GenPept-GenBank 76.0), as well as the Protein Kinase Catalytic Domain Data Base compiled by Hanks and Quinn (6), indicated that A6 showed no significant overall similarity with any previously identified proteins.

The protein kinase family, which includes more than 100 members of eukaryotic origin, all have a discrete catalytic domain that can be divided into 12 different subdomains (6). The conserved features within these subdomains help to define a consensus sequence for protein kinases. However, A6 did not possess any of the motifs commonly conserved among protein kinases as judged by using pattern- and profile-matching programs (University of Wisconsin Genetics Computer Group's Motifs, ProfileScan and ProfileGap) (4).

There have been previously reported proteins that exhibited kinase activity, although their amino acid sequence had little or no similarity to the catalytic domains of protein kinases. However, computer analysis of the A6 sequence failed to reveal significant matches with these atypical kinases such as BCR (21), HBx, the hepatitis B virus transactivator protein (28), and AceK, an isocitrate dehydrogenase kinase/phosphatase from *Escherichia coli* (16). For further comparative purposes, pairwise alignments between the A6 sequence, representatives of the major protein kinase subfamilies (6), and the atypical kinases BCR and HBx were generated by using the FASTA program (23). The RDF2 program was used to judge the statistical significance of the alignments (23). Alignment scores generated by the FASTP program are shown in Table 1. As judged with the aid of RDF2, scores of 80 and higher reflect significant similarity. Although no significant similarities were observed between the atypical kinases and any other protein kinase, relationships between various protein kinase subfamilies were readily apparent. Thus A6 appears to encode a unique atypical kinase.

In vitro tyrosine kinase activity of the A6 product. To establish that the A6 protein was a tyrosine kinase, lysates of cells expressing β-gal–A6 or β-gal alone were immunoprecipitated with anti-β-gal and immunocomplexes were incubated with [γ-^{32}P]ATP. SDS-PAGE analysis of immunoprecipitates from β-gal–A6 lysates revealed a ^{32}P-labeled 150-kDa species, consistent with the predicted molecular mass of β-gal–A6 (Fig. 4A). In contrast, no ^{32}P-labeled proteins were detected in immunoprecipitates from lysates expressing β-gal alone. These findings demonstrated in vitro kinase activity associated with the β-gal–A6 product. To identify the phosphorylated residues, we performed phosphoamino acid analysis. ^{32}P-labeled β-gal–A6 immunoprecipitates were resolved by SDS-PAGE, transferred to Immobilon-P, and excised for analysis. As illustrated in Fig. 4B, both pTyr and phosphoserine but not phosphothreonine were detected.

We next examined the ability of β-gal–A6 to phosphorylate an exogenous substrate. For this assay we utilized myelin basic protein and poly(Glu-Tyr), known in vitro substrates of a variety of tyrosine kinases. As shown in Fig. 5A and B, β-gal–A6 exhibited kinase activity toward both myelin basic protein and poly(Glu-Tyr). Moreover, phosphoamino acid analysis revealed that both were phosphorylated exclusively on tyrosine (Fig. 5C).

The lack of sequence similarity of A6 to typical kinases led us to compare its biochemical properties with those of bek/FGFR2, a member of the FGF tyrosine kinase receptor family, expressed like A6 as a β-gal fusion product, β-gal–bek (12). Requirements for the autophosphorylation of β-gal–A6 and β-gal–bek were determined by in vitro kinase assays with a series of buffer systems under different pH conditions and supplemented with various ions to determine cation dependency. Under the assay conditions used, β-gal–A6 and β-gal–bek displayed remarkable similarity. Both had optimal activity over a pH range of 6.5 to 7.4 and were inactive at pH 8.0. As shown in Fig. 6, β-gal–A6 and β-gal–bek kinase activities were each found to be dependent on the presence of either manganese or magnesium ions but not zinc or calcium ions. Both β-gal–A6 and β-gal–bek displayed higher incorporation of ^{32}P in the presence of manganese. These finding are consistent with the fact that tyrosine kinases, unlike serine/threonine kinases, prefer the divalent cation manganese over magnesium for kinase activity. Thus, despite its striking divergence from other tyrosine kinases, A6 exhibited very similar in vitro biochemical properties.

TABLE 1. Comparison of the predicted A6 amino acid sequence with those of the catalytic domains of representative protein kinases

Kinase	Alignment[a] of A6 sequence with:												
	Atypical kinases			Dual-specificity kinases					Tyrosine kinases			Serine/threonine kinases		
	A6	BCR	HBX	CLK	DPYK1	MCK1	MIK1	MKK1A	c-Src	MET	PDGFR-α	c-Mos	PKC-γ	RSK1-C
A6	1,730													
BCR	49	1233												
HBx	39	30	833											
CLK	29	32	34	1736										
DPYK1	27	23	31	109	1439									
MCK1	33	44	37	176	85	1621								
MIK1	28	27	32	101	113	164	1376							
MKK1A	30	54	31	109	107	195	207	1147						
c-Src	34	28	27	98	200	140	138	171	1330					
MET	29	33	34	47	113	111	138	171	447	1376				
PDGFR-α	41	32	36	82	160	167	97	137	395	414	1809			
c-Mos	55	32	37	77	119	146	118	146	171	183	155	1401		
PKC-γ	44	35	30	107	105	191	179	205	222	177	152	174	1395	
RSK1-C	69	29	33	132	130	195	36	183	153	126	152	123	267	1318

[a] The A6 sequence was compared with those of known protein kinases by using the FASTA program. The values represent the optimized alignment scores; values of 80 or higher indicate significant similarity. Designations for representative protein kinase catalytic domain sequences were taken from the protein kinase data base of Hanks and Quinn (8), except for BCR (21) and HBx (27).

A6 is a widely expressed gene. To examine the pattern of A6 expression, we performed Northern blot analysis on RNAs isolated from a variety of different human tissues. Under stringent hybridization conditions, the A6 cDNA probe revealed a single transcript of approximately 3.4 kb in various tissues, consistent with its size in M426 cells (Fig. 7). Thus the 3-kb cDNA isolated by expression cloning encompassed most if not all of the A6 transcript. The A6 mRNA was expressed at high levels in organs including the colon,

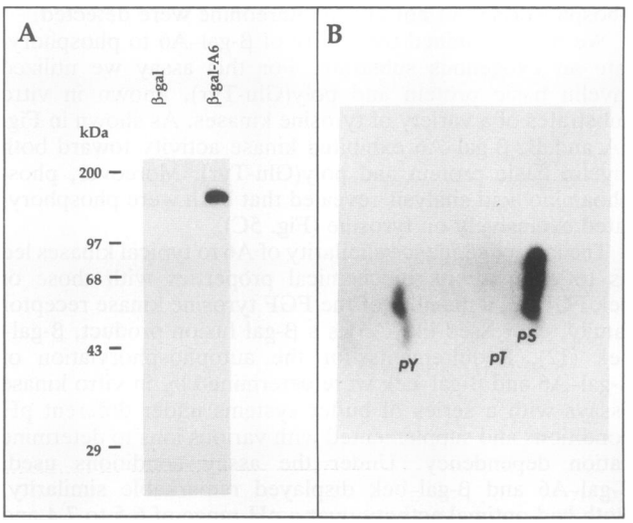

FIG. 4. In vitro kinase activity of β-gal–A6. (A) Bacterial lysates expressing either β-gal or β-gal–A6 fusion protein were immunoprecipitated with anti-β-gal antibody and assayed for kinase activity in the presence of [γ-^{32}P]ATP. ^{32}P-labeled products were resolved by SDS-PAGE (10% polyacrylamide) and transferred to an Immobilon-P membrane. (B) Phosphoamino acid analysis of β-gal–A6. The ^{32}P-labeled 150-kDa band was excised from the Immobilon-P membrane and acid hydrolyzed. Phosphoamino acids were resolved by two-dimensional electrophoresis prior to autoradiography for 24 h. Ninhydrin staining was used to identify the phosphoamino acid standards, i.e., phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY).

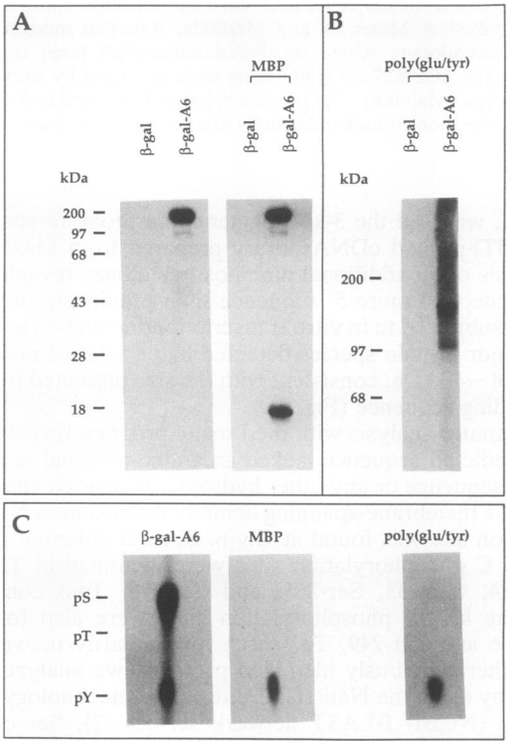

FIG. 5. Phosphorylation of exogenous substrates by β-gal–A6. (A and B) In vitro kinase assays were performed by incubating β-gal or β-gal–A6 immunocomplexes with either 10 μg of myelin basic protein (MBP) (panel A) or 2 μg of poly(Glu-Tyr) (panel B) in the presence of [γ-^{32}P]ATP. β-gal or β-gal–A6 with MBP was resolved by SDS-PAGE (12% polyacrylamide). β-gal or β-gal–A6 with poly-(Glu-Tyr) was resolved by using a 3 to 27% gradient gel. Gels were fixed, dried, and subjected to autoradiography. (C) Phosphoamino acid analysis of ^{32}P-labeled β-gal–A6, MBP, and poly(Glu-Tyr). Labeled bands corresponding to the 150-kDa β-gal–A6 band, 18-kDa MBP band, and the smeared poly(Glu-Tyr) band above β-gal–A6 were excised from each gel, and phosphoamino acids were resolved by one-dimensional electrophoresis. The position of standards, i.e., phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY), were identified by ninhydrin staining.

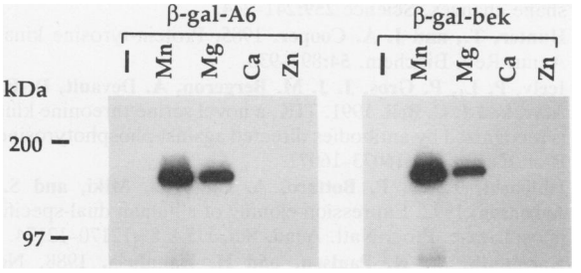

FIG. 6. Comparison of β-gal–A6 and β-gal–bek in vitro kinase activity. Bacterial lysates expressing β-gal–A6 or β-gal–bek were immunoprecipitated with anti-β-gal antibody. The immunocomplexes were incubated with [γ-^{32}P]ATP, kinase reaction buffer, and either 10 mM $MnCl_2$, 10 mM $MgCl_2$, 10 mM $ZnCl_2$, 10 mM $CaCl_2$, or no cation as indicated. Labeled proteins were resolved by SDS-PAGE (7.5% polyacrylamide), fixed, and dried prior to autoradiography.

testes, uterus, ovaries, prostate, and lungs. Lower levels were found in the brain, bladder, and heart, and there was no detectable A6 transcript in the liver.

An A6 probe readily detected related DNA fragments in genomic DNAs of species including human, murine, avian, amphibian, and fish (data not shown). Thus, A6 appears to be highly conserved in vertebrate evolution.

DISCUSSION

Expression cloning in prokaryotic systems has proven to be useful in the identification of novel protein kinases. This cloning approach is especially powerful since *E. coli* strains lack tyrosine kinases. Taking advantage of an assay that relies on enzymatic activity rather than sequence similarity, we sought to identify novel enzymes exhibiting tyrosine kinase activity but unrelated to previously identified members of the protein tyrosine kinase family. By screening an M426 cDNA library with anti-pTyr antibody, we identified two known tyrosine kinases, Src and Fyn, as well as a novel 150-kDa β-gal fusion protein.

Although anti-pTyr detection implied intrinsic activity of the expressed cDNA clone, the possibility of a cross-reacting epitope had to be excluded. For example, the protein kinase TIK, originally identified in a screening of a

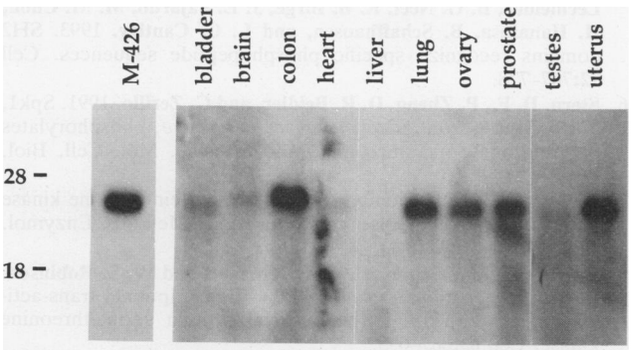

FIG. 7. Analysis of A6 transcript expression in human tissues. Total RNA extracted from the indicated adult human tissues, as well as poly(A)-containing RNA from M426 human embryo fibroblasts, were subjected to Northern blot analysis as described in Materials and Methods. The blot was hybridized with a ^{32}P-labeled A6 cDNA and subjected to autoradiography. Positions of the 28S and 18S rRNAs are indicated.

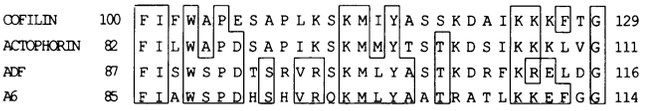

FIG. 8. Amino acid sequence alignment of A6 with actin-binding proteins. The predicted protein sequence of A6 was aligned with actin-depolymerizing factor (ADF), actophorin, and cofilin according to pairwise alignments generated by the BLASTP program. Residues identical to those at the same position in the A6 sequence are boxed.

mouse cDNA library with anti-pTyr antibody, failed to produce detectable amounts of pTyr when subsequently expressed in bacteria (11). These finding led the authors to conclude that the anti-pTyr antibody was reactive with sites other than pTyr. The lack of similarity between A6 and the catalytic domains of previously identified kinases made it conceivable that reactivity of A6 with anti-pTyr antibody could similarly have been due to an epitope other than pTyr. However, two-dimensional phosphoamino acid analysis demonstrated that A6 possessed phosphorylated tyrosine residues. In addition, this protein displayed tyrosine phosphorylation activity toward in vitro substrates such as myelin basic protein and poly(Glu-Tyr).

Until recently, protein kinases were known to phosphorylate only serine and threonine residues or tyrosine residues. However, within the last few years, additional protein kinases have been isolated which phosphorylate all three residues and have thus been termed dual-specificity protein kinases (19). A6 was shown to be phosphorylated on both tyrosine and serine residues, but it lacked the ability to phosphorylate serine residues in myelin basic protein. However, we cannot yet conclude that A6 exhibits dual specificity since the possibility remains that its phosphorylation of serine residues is attributed to a contaminating bacterial kinase.

Several additional features of the A6 sequence suggested that this enzyme may be involved with pathways regulating cell proliferation. There was a short stretch of significant similarity to the actin-depolymerizing factors isolated from evolutionarily diverse species (Fig. 8). Over a 30-amino-acid stretch from Phe-85 to Gly-114, A6 was found to be 77, 42, and 33% related to the actin-depolymerizing factors from *Lilium longiflorum*, *Acanthamoeba castellanii*, and chicken cofilin. It has been proposed that the corresponding sequence in cofilin (A6 Trp-88 to Met-99) is responsible for binding to actin and polyphosphoinositides (29). Therefore, A6 may be linked to cytoskeletal structure or phosphoinositide turnover. As for cytoskeletal structure, changes in cell shape have been associated with tyrosine phosphorylation of actin in *Dictyostelium discoideum* (9). Whether A6 plays a similar role in mammalian cells remains to be determined.

The predicted A6 protein sequence contained a number of consensus phosphorylation motifs for a number of different kinases including protein kinase C, casein kinase, and tyrosine kinases. These motifs suggest that A6 activity may be regulated by other kinases. To gain insight into potential A6 pathways, amino acid residues adjacent to tyrosine residues in the A6 sequence were compared with the phosphopeptide sequences predicted to interact with proteins having Src homology 2 (SH_2) domains (25). Interestingly, Tyr-68, Tyr-200, and Tyr-309 contained motifs that predicted possible interaction with phospholipase C-γ, Syp (SHPTP2N), and members of the Src family of kinases. Studies are presently under way to determine whether these interactions occur in vitro. Lastly, A6 appears to be a ubiquitous protein ex-

pressed in many major organ systems and is highly conserved phylogenetically.

Throughout evolution, eukaryotic organisms have acquired a need to respond to their surroundings. This need to respond to external stimuli has resulted in the development of extensive phosphorylation networks (17). The level of protein phosphorylation is governed by the combined actions of protein kinases and phosphatases, which in turn mediate physiological processes including growth, differentiation, and the unique functions characteristic of a given cell type. The fact that A6 lacks consensus kinase motifs but exhibits the functional activity of a kinase may reflect a convergence of function through evolution in which multicellular organisms have adapted to meet the multitude of requirements for function associated with higher organisms. Alternatively, A6 may represent a very highly divergent member of the tyrosine kinase family.

The association of tyrosine phosphorylation with the transforming activity of a number of oncogenes, as well as being the primary signal transduction mechanism for a large number of growth factors and cytokines, establishes an important role of these enzymes in cell growth and differentiation pathways. The unique structure of A6 would have prevented its detection by other screening techniques such as low-stringency hybridization or PCR. Studies are under way to determine the physiological function of the A6 kinase.

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