TIK, a Novel Serine/Threonine Kinase, Is Recognized by Antibodies Directed against Phosphotyrosine*

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We have isolated cDNAs encoding kinases from a murine pre-B cell line by screening a λgt11 cDNA expression library with anti-phosphotyrosine antibodies. One cDNA was identified to encode the previously isolated tyrosine kinase c-lyn. Among the remaining clones, we have characterized a cDNA encoding a novel kinase which we have designated TIK. Sequence analysis of this cDNA indicates that the TIK enzyme lacks the features thought to be conserved among protein tyrosine kinases. Although isolated on the basis of its reactivity with the anti-phosphotyrosine antibody, the TIK enzyme was found to have only serine and threonine kinase activity. The amino-terminal portion of the TIK protein contains a cdc2 phosphorylation consensus sequence. Three mRNA transcripts derived from the TIK gene are detected in a variety of adult murine tissues.

Molecules comprising cellular signal transduction networks provide a link from extracellular stimuli to the internal environment of the cell. Currently, attempts are being made to dissect the pathways that transduce signals controlling cellular growth and development. Some protein tyrosine kinases have been identified as growth factor receptors, clearly depicting these enzymes as mediators of cell proliferation and differentiation signals (1). Recent identification of serine/threonine kinases as downstream effectors of specific tyrosine kinases indicates that cellular signal transduction requires the coordinated action of a variety of kinase (2).

It has become apparent that the regulated expression of specific tyrosine kinases during hematopoiesis is crucial to normal hematopoietic development. Mutations at the white locus of the mouse, which has been identified to encode the tyrosine kinase c-hck (3), lead to developmental defects including severe macrocytic anemia and mast cell deficiency. In addition to playing a role in the transduction of signals controlling cell growth and development, some src-related tyrosine kinases have specialized functions in fully differentiated hematopoietic cells (4).

Enzymatically active tyrosine kinases undergo autophosphorylation, allowing their detection by antibodies to phosphotyrosine. Such antibodies have been successfully used in a variety of systems to screen cDNA expression libraries for cDNAs encoding functional tyrosine kinases (5–7). Using this method, we have isolated a number of cDNA clones from a pre-B-cell expression library. One cDNA was found to encode a kinase which appeared to be more closely related to serine/threonine kinases than tyrosine kinases. We present evidence here that although this novel kinase can be recognized by the anti-phosphotyrosine antibody, it is in fact a serine/threonine kinase.

MATERIALS AND METHODS

Isolation of cDNA Clones

cDNAs encoding anti-phosphotyrosine immunoreactive proteins were isolated from a λgt11 cDNA expression library generated from poly(A)+-selected RNA from the 70Z/3 cell line. 5 × 10⁶ plaques were screened by infecting the bacterial strain Y1090 with the bacteriophage and incubating at 42 °C for 4–6 h, overlaying with filters soaked in 10 mM 1-isopropropyl-β-D-thiogalactopyranoside, and incubating for an additional 6 h at 37 °C. Filters were then treated with polyclonal murine anti-phosphotyrosine antibody and anti-mouse antibody coupled to alkaline phosphatase. Positive clones were visualized through reaction with Nitro Blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. These clones were rescreened twice to homogeneity.

Positive clones were cross-hybridized to identify distinct clones by the following method: 1 μl of phage in solution at 10 pfu was spotted onto a plated bacterial lawn of Y1090, allowed to adsorb for 20 min at 25 °C, and incubated for 8–12 h at 37 °C. Nitrocellulose filters were overlayed for 4 min, denatured in 1.5 M NaCl, 5 M NaOH for 2 min, neutralized in 1.5 M NaCl, 0.5 M Tris-CI, pH 8, for 5 min, and rinsed in 0.2 M Tris-CI, pH 7.5, 2 × SSC for 30 s. Filters were baked at 80 °C under vacuum for 2 h. Filters were probed with DNA probes generated by random primer extension labeling of polymerase chain reaction-amplified cDNA inserts. Prehybridizations were carried out with 6 × SSC, 5 × Derhadt’s solution, 0.5% SDS; and denatured salmon sperm DNA at 0.1 mg/ml at 65 °C for 6 h. Hybridizations were performed using radiolabeled DNA labeled probe at 1–4 × 10⁶ cpm/ml for 12 h. Filters were washed in 2 × SSC at 22 °C for 5 min and once in 0.1 × SSC, 0.1% SDS at 22 °C for 15 min, then autoradiographed by exposure to Kodak XAR-5 x-ray film.

Sequencing of cDNA Clones

The cDNA inserts were cloned into the EcoRI site of the bacteriophage M13 (Pharmacia LKB Biotechnology Inc.), and single-stranded sequence analysis was carried out using the Sanger dideoxy chain termination method (25). Predicted amino acid sequences were compared with the proteins of the National Biomedical Research Foundation protein data bank using the RUNFASTP program.

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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) M65029.

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kδ, kilobase(s); Mops, 4-[N-(2-hydroxyethyl)amino]-2-ethanesulfonic acid.

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**RNA Isolation and Northern Blot Analysis**

Total RNA was prepared from cells or tissues by the method of Auffray and Rougeon (26). Poly(A)\(^+\) selection was carried out by passage of total RNA over oligo(dT)-cellulose, following the method of Jacobson (27). Aliquots of 4 \(\mu\)g of poly(A)\(^+\) RNA were electrophoretically separated. RNAs were transferred to Hybond-N membrane in 1% agarose gels containing 2.2 M formaldehyde, 20 mM Mops (pH 6.8), 1 mM EDTA, and 5 mM sodium acetate, and the electrophoretically separated RNAs were bashed for 2 h at 80 °C and treated with ultraviolet light for 5 min. Prehybridization was carried out 12-24 h in 5X SSPE, 50% formamide, 2 mM NaPi, pH 6.8, 5X Denhardt’s solution, 5% dextran sulfate, 0.5% SDS, and 250 \(\mu\)g/ml denatured nonhomologous DNA, at 42 °C. Hybridizations were performed using a random-primed \(^{32}\)P-labeled DNA insert at 1-2 \(\times\) 10\(^6\) cpm/ml at 42 °C for 12-24 h. Filters were washed to a final stringency of 0.1 X SSC, 0.1% SDS at 22 °C, and exposed to Kodak XAR-5 x-ray film.

**Bacterial Expression of the TIK cDNA**

The entire coding region of the TIK cDNA from the BamHI site (position 116) 21 base pairs upstream of the putative initiating methionine residue to position 1849 (146 base pairs to the stop codon) was subcloned into the BamHI site of the pET11c expression vector (28, 29). *Escherichia coli* pLysS containing this construct was typically induced to express the T7 gene 10 product-TIK fusion protein by growing the bacteria to an OD\(_\text{OD}_{600}\) of 0.6 in LB medium containing 50 \(\mu\)g/ml ampicillin, and adding 0.4 mM isopropyl-\(\beta\)-D-thiogalactopyranoside to the bacterial medium and growing for 2 h.

**Protein and Phosphoamino Acid Analysis**

*In Vitro Labeling Experiments*—*E. coli* pLysS bacteria containing the TIK sense or antisense pET11c construct were induced (as described above) to express the T7 gene 10 product-TIK fusion protein and lysed by freeze-thawing. Lysates were cleared by centrifugation, and the supernatants were immunoprecipitated with either PV20 (from ICN Biochemicals), IgG2bk (from Upstate Biotechnology, Inc.), MAb2G2 (generous gift of Dr. A. Raymond Frackelton, Jr., Brown University), or a polyclonal rabbit anti-phosphotyrosine antibody, in in 10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM sodium pyrophosphate, 500 \(\mu\)M ammonium vanadate, and 200 \(\mu\)g of phenylmethylsulfonyl fluoride per ml. The immunoprecipitates were assayed for kinase activity in 20 mM HEPES, pH 7.1, and 10 mM MnCl\(_2\) with 0.1 mCi/ml \([\gamma^{32}\text{P}]\)ATP for 30 min at 22 °C. The reaction products were resolved by 10% SDS-polyacrylamide gel electrophoresis, and the dried gels were exposed to Kodak XAR-5 film. Phosphoproteins were electroeluted for phosphoamino acid analysis by the method of Edwards et al. (30).

*In Vivo Labeling Experiments*—Bacteria harboring the TIK sense or antisense expression construct were grown to an OD\(_\text{OD}_{600}\) of 0.6 in LB medium containing 50 \(\mu\)g/ml ampicillin, and adding 0.4 mM isopropyl-\(\beta\)-D-thiogalactopyranoside to the bacterial medium and growing for 2 h. Induced bacteria were resolved by 10% SDS-PAGE and transferred to Immobilon P membranes. Blotted proteins were denatured in 6 M guanidinium chloride, 50 mM Tris, 50 mM dithiothreitol, and 2 mM EDTA, and then renatured overnight at 4 °C in 100 mM NaCl, 50 mM Tris, 2 mM dithiothreitol, 2 mM EDTA, and 0.1% (v/v) Nonidet P-40 (ph 7.5). Blots were then blocked with 5% (w/v) albumin in 30 mM Tris (ph 7.5) at room temperature for 1 h. The kinase assay was performed by incubating blocked blots in 30 mM Tris, 10 mM MgCl\(_2\), and 10 mM MnCl\(_2\) (pH 7.5), with 50 \(\mu\)Ci/ml \([\gamma^{32}\text{P}]\)ATP at room temperature for 30 min. Blots were washed twice with 30 mM Tris (pH 7.5), followed by 30 mM Tris (pH 7.5) and 0.05% Nonidet P-40 for 10 minutes at room temperature prior to autoradiography.

**RESULTS**

**Isolation of cDNA Clones**—We have used an antibody to phosphotyrosine to screen a Xgt11 cDNA expression library prepared with mRNA isolated from the pre-B cell line 70Z/3 (8). From the screening of 5 \(\times\) 10\(^5\) recombinant plaques we identified eight distinct clones encoding fusion proteins reacting with a polyclonal anti-phosphotyrosine antibody. Through cDNA sequence analysis, one cDNA was identified as the tyrosine kinase c-lyn (9), verifying the efficacy of this screening procedure. The mRNA sizes for the remaining seven clones were determined by Northern blot analysis using poly(A)\(^+\)-selected RNA from the 70Z/3 line (Fig. 1). In agreement with their designation as distinct gene products, these cDNAs hybridize to mRNAs of varying abundance and transcript size. It has been shown previously that the tyrosine kinase c-abl gene is expressed in 70Z/3 cells (8). None of our clones, however, were found to hybridize with a c-abl probe.

**Characterization of the TIK Kinase**—One novel kinase

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**FIG. 1.** Northern blot analysis showing mRNA sizes of the seven distinct cDNAs isolated from the 70Z/3 cell line. 5 \(\mu\)g of poly(A)\(^+\)-selected RNA was used for each lane of the gel and hybridized to a cDNA probe generated for each cDNA. The 18 and 28 S rRNAs are 1869 and 4712 nucleotides, respectively.

**FIG. 2.** Northern blot analysis of TIK mRNA in a variety of adult mouse tissues. Each lane contains approximately 5 \(\mu\)g of poly(A)\(^+\)-selected RNA isolated from each mouse tissue. The blot was hybridized to a TIK cDNA and murine \(\beta\)-actin probe. The 18 and 28 S RNAs are 1869 and 4712 nucleotides, respectively.
Fig. 3. The nucleotide and predicted amino acid sequence of the TIK cDNA. The cdc2 phosphorylation consensus sequence is boxed, and the residues making up the catalytic domain are underlined.

FIG. 3.

FIG. 4. A schematic representation of the TIK kinase. Serine/threonine and tyrosine kinase subdomain consensus sequences are indicated by Roman numerals. X, any amino acid. Boxed amino acids represent conserved residues.

FIG. 5. In vivo labeling of the TIK kinase. Lysates of E. coli pLysS expressing the TIK fusion protein in either the sense (A) or antisense (B) orientation in the presence of [35S]methionine. Lane 1, [35S]methionine added at the time of induction and bacteria harvested after 1.5 h; lane 2, 0.5 h postinduction and bacteria harvested after 1 h.

which we designated TIK, for anti-phosphotyrosine immunoreactive kinase, was selected for further study. A 2.1-kb TIK cDNA hybridizes to three mRNAs of 6, 4, and 2.5 kb in the 70Z/3 line (Fig. 1). Transcripts hybridizing to the TIK cDNA are also detected in murine heart, lung, brain, kidney, testes, thymus, and bone marrow (Fig. 2). There are tissue-specific differences in the ratios of the three TIK transcripts; for example, in both lung and heart tissue the 4-kb mRNA is predominant over the 6- and 2.5-kb mRNAs (Fig. 2). The 2.5-kb mRNA is expressed at high levels in testes tissue, suggesting that TIK may play a role in spermatogenesis. High stringency hybridization of the complete TIK cDNA to 70Z/3 DNA digested with the restriction endonucleases HindIII, BamHI, and KpnI produced a very simple pattern of bands, indicating that the three mRNAs originate from a single gene (data not shown).

The nucleotide sequence of the TIK cDNA is shown in Fig. 3. The sequence contains an open reading frame of 517 amino acids ending at position 1701. The putative initiating methionine residue at nucleotide position 147 is preceded by one
Characterization of the TIK Kinase

Fig. 6. A, in vitro kinase reactions of the TIK fusion protein in bacterial lysates. Lysates of E. coli pLysS expressing the TIK fusion protein in either the sense (lane 2) or antisense (lane 1) orientation were immunoprecipitated with an anti-phosphotyrosine antibody and assayed for kinase activity in the presence of [γ-32P]ATP prior to SDS-polyacrylamide gel electrophoresis. B, phosphoamino acid analysis of electroeluted products from panel A. The positions of the phosphoamino acid standards are indicated as follows: pS, phosphoserine; pT, phosphothreonine; and pY, phosphotyrosine. C, in vivo labeling of the TIK fusion protein. E. coli pLysS expressing the TIK fusion protein in either the sense (lane 1) or antisense (lane 2) orientation were grown in the presence of [32P]orthophosphate. Lysates from these bacteria were resolved on SDS-polyacrylamide gel electrophoresis and autoradiographed. D, phosphoamino acid analysis of electroeluted products from panel C. E, kinase renaturation assay of the TIK kinase. Lysates of E. coli pLysS expressing the TIK fusion protein in either the sense (lane 1) or antisense (lane 2) orientation were resolved by SDS-PAGE and transferred to an Immobilon P membrane. The blotted proteins were denatured and reassembled as described under “Materials and Methods” and assayed for kinase activity in the presence of [γ-32P]ATP. The blot was then washed and autoradiographed. F, phosphoamino acid analysis of the protein blotted onto Immobilon P shown in panel E.

termination codon, and the sequence surrounding this ATG site conforms to the consensus predicted by Kozak’s rules for ribosome binding (10). The TIK protein predicted from the cDNA sequence would have a molecular mass of 58,573 Da, with an isoelectric point of 8.38.

The presumed catalytic region of the TIK enzyme is contained within amino acid residues 252-505. When compared with the NBRF-PIR(r) data base using the Lipman and Pearson algorithm (11), TIK was found to share a high degree of amino acid sequence identity with serine/threonine kinases. The highest degree of identity was found with the cdc2 kinase (27% identity in a 185-amino acid overlap). In general, the catalytic domain of protein kinases contains conserved regions designated by Hanks et al. (12) as subdomains I–XI. As the TIK kinase was cloned by virtue of its immunoreactivity with the anti-phosphotyrosine antibody, we expected its conserved subdomains would most closely resemble those of the tyrosine kinase family. The primary sequences of subdomains VI and VIII are thought to be indicative of the hydroxyamino acid specificity of the kinase. As illustrated in Fig. 4, these regions in TIK match more closely the serine/threonine rather than the tyrosine kinase consensus sequence.

Other features of the TIK kinase which may be of significance lie amino-terminal to the catalytic domain. This region of the predicted TIK polypeptide (residues 1-252) shares little homology with previously identified serine/threonine or tyrosine kinases. The primary sequence does not provide a clear indication of the subcellular localization of the TIK enzyme. The kinase has no apparent transmembrane domain, nor does it have a myristylation site at residue 2, which is typically found in tyrosine kinases targeted to membrane surfaces (13). The TIK protein does not have an SH-2 domain (14), which is found in non-receptor-type tyrosine kinases and other enzymes of the signal transduction network.

The kinase recognition sequence for cdc2 phosphorylation, Ser/Thr-Pro-Xaa-Arg/Lys (15), is found at residues 157-161 of the TIK protein (Fig. 3). This consensus sequence is followed by several serine and threonine residues, in particular two clusters of serine residues.

Kinase Activity of TIK—The observation that the catalytic domain of the TIK kinase resembled more closely the catalytic domains of serine/threonine kinases than those of tyrosine kinases prompted us to investigate the biochemical activity of this enzyme. The TIK kinase was expressed in bacteria as a fusion protein, using a cDNA construct in which the coding region of the T7 gene 10 product is fused in frame with the 5’ end of the entire TIK coding region. In vitro transcription and translation of this TIK fusion protein produced a polypeptide of approximately 67 kDa (data not shown). The expected molecular mass of the TIK fusion protein was approximately 63 kDa, so it appeared to be migrating anomalously in the SDS-PAGE system. Other phosphoproteins also migrate to unexpected apparent molecular masses in SDS-PAGE systems, for example the E1A protein of adenovirus (16) and the NS protein of vesicular stomatitis virus (17).

When intact bacteria expressing the TIK fusion protein were metabolically labeled with [35S]methionine, a 67-kDa polypeptide was produced that can be immunoprecipitated with antibodies to phosphotyrosine (Fig. 5). Bacteria expressing an identical TIK antisense construct did not produce this polypeptide, as expected. The TIK fusion protein was resolved by SDS-PAGE and transferred to an Immobilon P membrane. The blotted proteins were denatured and reassembled as described under “Materials and Methods” and assayed for kinase activity in the presence of [γ-32P]ATP. The blot was then washed and autoradiographed. F, phosphoamino acid analysis of the protein blotted onto Immobilon P shown in panel E.
polypeptide, confirming the identity of this 67-kDa polypeptide with the TIK fusion protein produced by *in vitro* transcription and translation. The immunoprecipitated 67-kDa protein was found to have an associated kinase activity when incubated with [*32P*]ATP in an *in vitro* kinase assay (Fig. 6A). Phosphoamino acid analysis shows that this protein contains phosphoserine and phosphothreonine, but not phosphotyrosine (Fig. 6B). In an experiment in which bacteria expressing the TIK fusion protein were labeled *in vivo* with [*32P*]orthophosphate, a labeled 67-kDa protein was immunoprecipitated with anti-phosphotyrosine antibodies (see Fig. 6C). Phosphoamino acid analysis of this 67-kDa band showed phosphoserine and phosphothreonine, but no phosphotyrosine (see Fig. 6D).

Although these experiments strongly suggest that the 67-kDa TIK fusion protein has intrinsic serine/threonine kinase activity, it remained formally possible that this activity is attributable to an associated bacterial kinase. To rule out this possibility, we performed a kinase renaturation assay. In this procedure, lysates from bacteria expressing the TIK fusion protein were separated by gel electrophoresis, blotted to an Immobilon P membrane, induced to renature as described by Ferrell and Martin (18), and incubated with [*32P*]ATP. The renatured 67-kDa TIK fusion protein incorporates radioactively labeled phosphate (Fig. 6E). Lysates from bacteria expressing the TIK antisense construct did not show this activity. Phosphoamino acid analysis of the renatured TIK kinase showed that it contains phosphoserine and phosphothreonine (see Fig. 6F), confirming that the TIK protein has intrinsic serine/threonine kinase activity.

**DISCUSSION**

We have cloned and characterized a novel kinase, TIK, from the pre-B cell line 70Z/3. TIK has intrinsic serine and threonine kinase activity, both in *in vitro* kinase assays and *in vivo* in bacteria. Under no conditions was this kinase found to have tyrosine phosphorylating ability. This finding is in agreement with our observation that the catalytic domain of TIK contains residues thought to be conserved among serine/threonine kinases, but not tyrosine kinases (12) (Fig. 4). When compared with the proteins of the NBRF database, the TIK kinase was found to share the highest degree of identity with the serine/threonine kinase cdc2. Members of the cdc2 protein kinase family typically contain a short sequence of strong homology near subdomain XI of their catalytic domains. Although the region of shared identity between the TIK and cdc2 kinases is found in the catalytic domain, it does not include the sequences surrounding subdomain XI.

The TIK cDNA hybridizes to three mRNA transcripts in all mouse tissues analyzed (Fig. 2). Other single-copy kinase genes have been shown to give rise to multiple mRNAs through alternative splicing of a single mRNA species or differential promoter usage (19–24). These additional transcripts encode proteins differing in specific domains. While the size difference of the TIK transcripts could represent varying untranslated regions, it is possible that these mRNAs give rise to three isoforms of this enzyme.

Despite its lack of phosphotyrosyl residues, the TIK protein can be detected and immunoprecipitated with three different monoclonal anti-phosphotyrosine antibodies and a polyclonal anti-phosphotyrosine antibody. A possible explanation for this apparent paradox is that these antibodies are cross-reacting with phosphoserine or phosphothreonine. However, detection of the 67-kDa TIK fusion protein band in anti-phosphotyrosine immunoblots of lysates from TIK expressing cells was blocked by competition with 5 mM phosphotyrosine, but not with phosphoserine or phosphothreonine (data not shown). This observation suggests that an epitope resembling phosphotyrosine may be encoded by the TIK primary sequence. If indeed such an epitope does exist it may play a role in the regulation of the biochemical and biological properties of the TIK kinase. The TIK epitope detected by the anti-phosphotyrosine antibodies may represent a binding site for molecules which specifically recognize phosphotyrosyl residues and could therefore allow the TIK kinase to gain access to signal transduction molecules which normally communicate via phosphotyrosine/protein interactions. Experiments designed to test this hypothesis are currently underway.

Use of the anti-phosphotyrosine antibody has been considered a specific screen for functional tyrosine kinases (5–7). Our observation that the TIK kinase reacts with such antibodies does not possesses tyrosine phosphorylating ability indicates that this screening procedure also unexpectedly detects novel serine/threonine kinases.

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