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NOTES

Novel Tyrosine Kinase Identified by Phosphotyrosine Antibody Screening of cDNA Libraries

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In an attempt to clone protein tyrosine kinases, antiphosphotyrosine antibodies were used to screen λgt11 cDNA expression libraries. By this method, a 2.5-kilobase cDNA encoding a novel tyrosine kinase was isolated from a mouse liver cDNA library. This new gene is most closely related to the receptor tyrosine kinases *ret*, *fms*, and *kit*.

The transduction of cellular genes encoding protein tyrosine kinases into acutely transforming retroviruses and the association of protein tyrosine kinase activity with a number of growth factor receptors (6–8, 12, 14, 22, 25) have implicated tyrosine kinases in the control of cellular growth. Therefore, the isolation and characterization of these genes allow for the identification of proteins which may be important in cellular growth control pathways. While a number of novel tyrosine kinases have been identified by screening cDNA and genomic libraries at low stringency with probes homologous to the catalytic domains of already identified protein tyrosine kinases (9, 18, 21), we sought to develop new methods for identifying these genes while simultaneously allowing for large-scale production of identified protein kinases and confirmation of the catalytic activity of the cloned genes.

In an effort to identify cellular targets of tyrosine kinases, antibodies directed against phosphotyrosine have been produced (10, 11, 26). We used such antibodies to screen λgt11 cDNA expression libraries, which allow for the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible production of cDNA-encoded proteins fused to β-galactosidase. A tyrosine kinase produced from the λ vector would, if active, phosphorylate either itself (autophosphorylation) or bacterial proteins, allowing for the detection of phosphotyrosine-containing proteins picked up on nitrocellulose filters. Since there is no detectable tyrosine kinase activity in bacteria, we expected any positive signal to come from the expression of introduced cDNA clones (16, 20). To screen for protein tyrosine kinases, we used a cDNA library constructed from poly(A)$^+$ RNA from adult mouse liver (1). A total of 500,000 plaques were screened by infecting the bacterial strain Y1090 with bacteriophage, incubating at 42°C for 4 h, overlaying with nitrocellulose filters coated with 10 mM IPTG, and incubating further for 6 h at 37°C. The filters were then processed with antiphosphotyrosine antibody and ^{125}I-protein A as described elsewhere (15). Figure 1A shows an autoradiograph of filters from two plates, both infected with a positive clone which we isolated from the mouse liver cDNA library by this method. The left side of Fig. 1A shows the results obtained with antibody prepared against phosphotyramine (11), while the right side shows a filter probed with antiphosphotyrosine antibody made from the injection of rabbits with bacterially produced v-*abl* protein (26). The two antibodies gave identical signals.

To characterize the fusion protein produced by the recombinant bacteriophage, 10 ml of a 1:100 dilution of Y1090 bacteria was shaken at 30°C for 6 h with the phage carrying the positive clone (in the presence of 10 mM IPTG to induce protein production). The bacteria expressing the putative tyrosine kinase were lysed by vortexing with glass beads in RIPA buffer (0.1% sodium dodecyl sulfate, 1% Triton X-100,

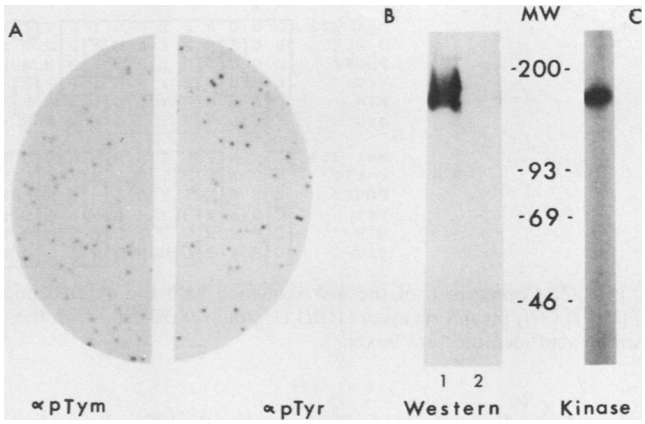

FIG. 1. (A) Probing of λgt11 nitrocellulose filters with antiphosphotyrosine (pTyr) and antiphosphotyramine (pTym) antibodies. Filters were exposed for 6 h after detection with ^{125}I-protein A. (B) Antiphosphotyrosine Western blot (immunoblot) of infected bacteria. A 1:100 inoculation of a Y1090 overnight culture was shaken with a sample of the positive phage at 30°C for 6 h. Infected bacteria were lysed, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and probed with antiphosphotyrosine antibody. (C) In vitro kinase assay. Infected bacteria were prepared as described for panel B, and lysates were immunoprecipitated with polyclonal antibody against β-galactosidase. After being washed, the immunoprecipitates were incubated in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4)–10 mM $MnCl_2$–0.1 μM [γ-^{32}P]ATP. Phosphorylation was predominantly on serine (data not shown), presumably because of the presence of contaminating serine kinases, with only a trace of detectable phosphotyrosine.

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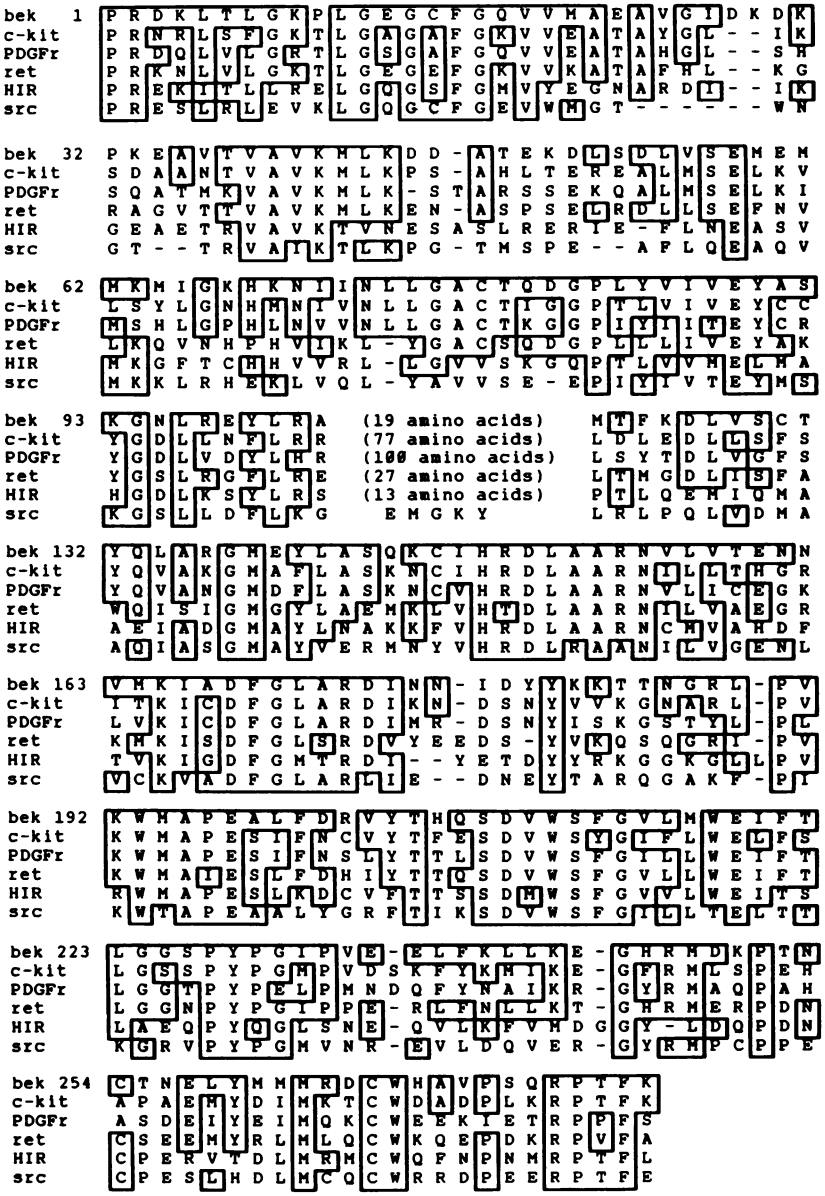

FIG. 2. Comparison of the *bek* sequence with the cytoplasmic domains of human c-*kit* (28), platelet-derived growth factor receptor (PDGFr) (27), insulin receptor (HIR) (7), and *ret* (23) and with the catalytic domain of $p60^{src}$ (24). Numbers indicate the amino acid of *bek*. Amino acid identities are boxed.

1% sodium deoxycholate, 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 10 mM Tris hydrochloride [pH 7.4]). The protein extracts from control bacteria and from λ-infected Y1090 cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electrophoretically transferred to nitrocellulose. No phosphotyrosine-containing proteins were detected in control bacteria (Fig. 1B), explaining the lack of significant background in our screening system. While a number of phosphorylated bacterial proteins were detected at low levels in bacteria expressing the positive clone (which we will refer to hereafter as *bek* for bacterially expressed kinase), the predominant protein detected in the immunoblot was a 160-kilodalton protein corresponding to the β-galactosidase fusion protein. Immunoprecipitation of the same protein lysates with anti-β-galactosidase antibody, followed by phosphorylation in vitro with [γ-^{32}P]ATP, revealed a protein of the same size (Fig. 1C).

To further characterize the isolated clone, we excised the cDNA insert with *Eco*RI and hybridized the cDNA to a panel of tyrosine kinase oncogenes, including *erbB*, *yes*, *ros*, *fms*, *src*, *fgr*, and *fps*. Since the 2.5-kilobase *bek* insert did not hybridize to DNA from any of these known tyrosine kinases (data not shown), we subcloned *bek* into M13 for nucleotide sequencing. Sequencing showed the newly isolated gene to be similar to but distinct from previously identified tyrosine kinases, with particular similarity to the receptor-type kinases *kit*, platelet-derived growth factor receptor, *fms* (CSF1 receptor), *ret*, and the insulin receptor (ranging from 35 to 55% identity at the amino acid level in the homologous regions of the kinase domain) (Fig. 2). The *bek* insert encodes a single major open reading frame extending

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          10        20        30   □    40        50        60        70        80        90       100       110       120
ProArgAspLysLeuThrLeuGlyLysProLeuGlyGluGlyCysPheGlyGlnValValMetAlaGluAlaValGlyIleAspLysAspLysProLysGluAlaValThrValAlaVal
CCCAGAGATAAGCTGACGCTGGGCAAACCCCTGGGGGAAGGTTGCTTCGGGCAAGTAGTCATGGCTGAAGCAGTGGGAATCGATAAAGACAAACCCAAGGAGGCGGTCACCGTGGCAGTG

          130       140       150       160       170       180       190       200       210       220       230       240
41 LysMetLeuLysAspAspAlaThrGluLysAspLeuSerAspLeuValSerGluMetGluMetMetLysMetIleGlyLysHisLysAsnIleIleAsnLeuLeuGlyAlaCysThrGln
   AAGATGTTGAAAGATGATGCCACAGAGAAGGACCTGTCTGATCTGGTATCAGAGATGGAGATGATGAAGATGATTGGGAAACATAAGAACATTATCAACCTCCTGGGGGCCTGCACGCAG

          250       260       270       280       290       300       310       320       330       340       350       360
81 AspGlyProLeuTyrValIleValGluTyrAlaSerLysGlyAsnLeuArgGluTyrLeuArgAlaArgArgProProGlyMetGluTyrSerTyrAspIleAsnArgValProGluGlu
   GATGGACCTCTCTACGTCATAGTTGAATATGCATCGAAAGGCAACCTCCGGGAATACCTCCGAGCCCGGAGGCCACCTGGCATGGAGTACTCCTATGACATTAACCGTGTCCCCGAGGAG

          370       380       390       400       410       420       430       440       450       460       470       480
121 GlnMetThrPheLysAspLeuValSerCysThrTyrGlnLeuAlaArgGlyMetGluTyrLeuAlaSerGlnLysCysIleHisArgAspLeuAlaAlaArgAsnValLeuValThrGlu
    CAGATGACCTTCAAGGACTTGGTGTCCTGCACCTACCAGCTGGCTAGAGGCATGGAGTACTTGGCTTCCCAAAAATGTATCCATCGAGATTTGGCTGCCAGAAACGTGTTGGTAACAGAA

          490       500       510       520       530       540   ▼   550       560       570       580       590       600
161 AsnAsnValMetLysIleAlaAspPheGlyLeuAlaArgAspIleAsnAsnIleAspTyrTyrLysLysThrThrAsnGlyArgLeuProValLysTrpMetAlaProGluAlaLeuPhe
    AACAATGTGATGAAGATAGCAGACTTTGGCCTGGCCAGGGATATCAACAACATAGACTACTATAAAAAGACCACAAATGGGCGACTTCCAGTCAAGTGGATGGCTCCTGAAGCCCTTTTT

          610       620       630       640       650       660       670       680       690       700       710       720
201 AspArgValTyrThrHisGlnSerAspValTrpSerPheGlyValLeuMetTrpGluIlePheThrLeuGlyGlySerProTyrProGlyIleProValGluGluLeuPheLysLeuLeu
    GATAGAGTTTACACTCATCAGAGCGATGTCTGGTCCTTCGGGGTGTTAATGTGGGAGATCTTTACTTTAGGGGGCTCACCCTACCCAGGGATTCCCGTGGAGGAACTTTTTAAGCTGCTC

          730       740       750       760       770       780       790       800       810       820       830       840
241 LysGluGlyHisArgMetAspLysProThrAsnCysThrAsnGluLeuTyrMetMetMetArgAspCysTrpHisAlaValProSerGlnArgProThrPheLysGlnLeuValGluAsp
    AAAGAGGGACACAGGATGGACAAGCCCACCAACTGCACCAATGAACTGTACATGATGATGAGGGATTGCTGGCATGCTGTACCCTCACAGAGACCCACATTCAAGCAGTTGGTCGAAGAC

          850       860       870       880       890       900       910       920       930       940       950       960
281 LeuAspArgIleLeuThrLeuThrThrAsnGluGluTyrLeuAspLeuThrGlnProLeuGluGlnTyrSerProSerTyrProAspThrSerSerSerCysSerSerGlyAspAspSer
    TTGGATCGAATTCTGACTCTCACAACCAATGAGGAATACTTGGATCTCACCCAGCCTCTCGAACAGTATTCTCCTAGTTACCCCGACACAAGTAGCTCTTGTTCTTCAGGGGACGATTCT

          970       980   ●   990       1000  ●1010     1020      1030
321 ValPheSerProAspProMetProTyrGluProCysLeuProGlnTyrProHisIleAsnGlySerValLysThrOP
    GTGTTTTCTCCAGACCCCATGCCTTATGAACCCTGTCTGCCTCAGTATCCACACATAAACGGCAGTGTTAAAACATGA
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FIG. 3. Nucleotide sequence of *bek* cDNA. Nucleotide and amino acid sequences are shown and are numbered with the beginning of the *bek* clone as amino acid 1. Symbols: ▼, potential site of tyrosine autophosphorylation; □, start of the consensus for nucleotide binding; ●, tyrosines located near the C terminus of the protein which are potential negative regulatory sites.

1,040 nucleotides with 1.5 kilobases of noncoding information, including a poly(A) tail (data not shown). The predicted amino acid sequence of the *bek* protein includes an ATP-binding site at lysine 41, 21 residues downstream from the consensus Gly-X-Gly-X-X-Gly (13); a potential site of tyrosine autophosphorylation (Tyr-181), corresponding to the Tyr-416 of $p60^{src}$; and the sequence AAR (amino acids 152 to 154) found in several tyrosine kinases thus far sequenced (Fig. 3). It is noteworthy that the predicted amino acid sequence of *bek* includes a number of tyrosine residues near the carboxyl terminus of the protein, since tyrosines located in similar positions in the c-*fms* protein and in $p60^{src}$ have been implicated in the negative regulation of these kinases (3–5, 19). These putative negative regulatory tyrosines are located near the extreme carboxyl terminus of the *bek* protein, 50 residues C-terminal of the end of the catalytic domain (as defined by homology with $p60^{src}$). The presence of a 19-amino-acid insertion in the predicted amino acid sequence of the catalytic domain of *bek* (Fig. 3) suggests that this kinase may be a receptor. Members of the platelet-derived growth factor receptor family have relatively long amino acid insertions within their catalytic domains (e.g., 77 amino acids in the case of *kit*) (2, 17, 28), whereas the insulin receptor has an insertion of 13 amino acids in this region (relative to $p60^{src}$) (7, 24). There are no such insertions in the nonreceptor tyrosine kinases. The insertion sequence in *bek* is distinct from other insertion sequences thus far characterized (2, 7, 12, 17, 23, 27, 28). We cannot firmly conclude, however, that *bek* is a receptor for an unknown ligand, because the fusion of the *bek* protein to β-galactosidase occurred just 3′ of the expected location of a transmembrane domain (by homology). Analysis of the portion of *bek* sequenced revealed no obvious hydrophobic regions which could serve as a membrane anchor.

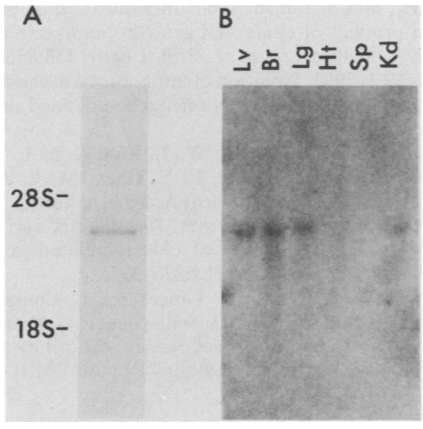

FIG. 4. Northern analysis of *bek* RNA. (A) RNA from adult mouse liver was poly(A)$^+$ selected, run on a formaldehyde gel, transferred to nitrocellulose, and probed with the full-length *bek* clone. (B) Total RNA from liver (Lv), brain (Br), lung (Lg), heart (Ht), spleen (Sp), and kidney (Kd) extracted from adult mice and probed with the *bek* cDNA.

Expression of the *bek* gene was examined by Northern analysis of total RNA from a number of adult mouse tissues. A single transcript of 4.3 kilobases was detected in RNA from liver (Fig. 4A), lung, brain, and kidney but was absent from heart and spleen (Fig. 4B).

The isolation of tyrosine kinases by using phosphotyrosine antibody to screen cDNA expression libraries requires that the kinase be active in bacteria. The tyrosine kinase activity of receptors can be activated by the binding of ligand. In the absence of ligand, the activation of tyrosine kinase activity may require structural alterations such as the truncation of the ligand-binding domain seen in the activation of c-*kit* and the epidermal growth factor receptor upon viral transduction (2, 6). Similarly, the removal of a putative ligand-binding domain of *bek* may have activated its kinase activity, allowing the detection of this clone in our screening system. It will be interesting to see whether the *bek* clone will transform cultured cells when expressed from retroviral vectors.

The ability to isolate clones on the basis of catalytic activity from an inducible expression vector allows the immediate production of large amounts of protein for biochemical characterization and for the production of antibod-

ies. In the case of *bek*, this will allow us to study a widely distributed and potentially interesting new receptor.

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ADDENDUM IN PROOF

The *bek* gene is very closely related to the *flg* gene isolated from a human endothelial cell cDNA library by Ruta et al. (Oncogene 3:9–15, 1988).

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