Isolation and Characterization of a Novel Receptor-type Protein Tyrosine Kinase (hek) from a Human Pre-B Cell Line*

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In this report we describe the identification and characterization of a novel tumor-associated receptor-type tyrosine kinase (hek). We produced a monoclonal antibody (III.A4) that detected a novel glycoprotein on the immunizing pre-B cell acute lymphoblastic leukemia cell line (LK63). This antigen was shown to be expressed sporadically on hemopoietic tumor cell lines and on ex vivo tumors. However, using antibody staining, the molecule was undetectable on normal tissues. Further biochemical characterization showed this molecule (hek) to be a phosphoprotein. This observation taken together with the tumor-associated nature of hek expression suggested that hek might be a receptor-type protein tyrosine kinase. This was demonstrated by affinity purification of hek. In in vitro kinase experiments the purified hek protein was autophosphorylated on tyrosine and also mediated tyrosine phosphorylation of casein. Purified hek was subjected to N-terminal amino acid sequence analysis which showed that hek had a unique N terminus. Amino acid sequence determination of peptides from a V8 protease digest of hek yielded one 21-amino acid stretch of sequence which is identical to the W/W mouse but possesses a normal c-kit gene.

The other line of evidence for a critical role of tyrosine kinase proteins in growth control came from the study of viral oncogenes (16, 17). These genes were shown to be directly involved in growth dysregulation by the introduction of DNA encoding these genes into murine fibroblasts. These oncogenes have been shown to have close cellular homologues (proto-oncogenes). One of the first identified oncogenes was v-src, the cellular homologue of which (c-src) is the prototypic representative of the family of cytoplasmic tyrosine kinases which, after myristylation, become associated with the inner leaf of the cell membrane (18). Within the hemopoietic system a number of lineage-restricted src-like kinases have been identified (19). For example the T cell-associated src-like kinase, lck, has been shown to associate independently with both the CD4 and CD8 transmembrane glycoproteins to form a signaling complex (20, 21). In contrast, v-erbB and v-fms, like their cellular homologues the epidermal growth factor receptor and colony-stimulating factor-1 receptor, respectively, are transmembrane molecules encoding the entire signal transduction machinery in a single polypeptide (1, 17).

Analysis of the amino acid sequences of these proteins has revealed conserved structural motifs within the catalytic domains (5). Both tyrosine kinase proteins have a consensus GXGXXG sequence which is found in many nucleotide-binding proteins (5). Some conserved sequence motifs are shared by both types of kinase whereas others are specific for the tyrosine or the serine-threonine kinase subgroups (5). The tyrosine kinases, although having regions of sequence conservation specific to this family, can be subdivided further according to the structural features of the regions 5' to the catalytic domain (1, 4-7).

In this report we describe a novel cell surface glycoprotein which is readily detected by the III.A4 monoclonal antibody on sporadic cell lines and tumor specimens but not on normal cells. We show that the III.A4 antibody identifies a novel human receptor-type tyrosine kinase. Purification of this protein from a pre-B acute lymphoblastic leukemia cell line, LK63, and amino acid sequencing identified this molecule as a member of the eph/eln family of tyrosine kinases (22-24). We assigned this molecule the provisional name hek (human eph/eln-like kinase). The possible role of hek in the neoplastic process is discussed.

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TABLE I

Cell lines tested for expression of the hek antigen

Hemopoietic cell lines analyzed by indirect immunofluorescence and flow cytometric analysis. Adherent cell lines were cultured on glass cover slips and analyzed by staining of the cultured cells in situ using indirect immunofluorescence.

Hemopoietic cell lines

- Pre-B: LK63, Lila-1, Reh, Nalm-1, FAKEM
- B cell: Raji, Daudi, RAMOS, U266, BALL-1
- T cell: HSB2, MOLT4, HPB-ALL, PEER, Jurkat, JM
- Myelomonocytic: HL60, U937, RC2a, THP-1, KG-1
- Erythroid: K562
- Megakaryocytic: HEL

Epithelial cell lines

- A51, HT29, LIM1215, ST6, LIM1899, COLO205, LIM1863
- Other: Embryonic fibroblasts, bone marrow stromal cells, umbilical vein endothelial cell, synovial cells, melanoma cell lines MM96 and C32

MATERIALS AND METHODS

Cell Lines

The LK63 and Lila-1 cell lines were established in culture from primary isolates of bone marrow and peripheral blood of two patients with acute lymphoblastic leukemia. Both had pre-B cell phenotypes and cytogenetic features characteristic of pre-B cell acute lymphoblastic leukemia, including the t:1:13 translocation (25). Details of these studies will be published elsewhere. Other hemopoietic lines analyzed in this study are listed in Table I. Nonhemopoietic cell lines included two melanoma lines and a number of epithelial lines A431, COLOHT29, COLOSTG, LIM1899, LIM1863, and COLO205, which were the gift of Dr. R. Whitehead, Ludwig Institute, Melbourne Branch. Normal human skin fibroblasts, umbilical vein endothelial cells, and synovial cells were also tested.

Monoclonal Antibodies

The III.A4 monoclonal antibody (IgG1, κ) was prepared by intraperitoneal immunization of a BALB/c mouse with LK63 cells. Spleen cells from this mouse were fused with NS-1 cells to form hybridomas. Supernatants of the resulting hybridomas were screened on LK63 cells by indirect immunofluorescence (see below) and positive clones isolated. The III.A4 antibody clone reacted strongly with LK63 but not with other hemopoietic cell lines. Other monoclonals used included: FM63 (CD19), B1 (CD20), IaT (CD36), W6/32 (major histocompatibility complex l), OKT8 (CD8) and PHM6 (CD10/CALLA).

Tumor Samples by Indirect Immunofluorescence

Fresh tumor material was obtained from biopsy specimens in accord with procedures approved by Institutional Ethical Review Boards. Single cell suspensions were prepared and the samples applied to Ficoll-Hypaque gradients. The buoyant density cells were isolated and cell surface phenotypic analysis performed.

Cells (10^6) were mixed with appropriate dilutions of monoclonal antibodies for 30 min on ice. The cells were washed and mixed with fluorescein-labeled F(ab)2 fragments of sheep anti-mouse immunoglobulin antibody and held for 20 min on ice. The samples were washed, fixed in 1% formalin in phosphate-buffered saline, and analyzed on a FACScan II cell sorter (Becton Dickinson, Mountain View, CA).

Immunoperoxidase Staining of Tissue Sections

Frozen sections were cut at 3 μm, placed on gelatin-coated slides, and fixed in acetone for 15 min and air dried. Sections were stained with III.A4 and control antibodies using the peroxidase-anti-peroxidase immunoperoxidase method (26).

Cell Labeling and Immunoprecipitation

- Labeling—Cell surface proteins were labeled with 35S-methionine. Cells were starved in methionine-free RPMI 1640, 5% dialyzed fetal calf serum for 2 h. In some experiments, the cells were labeled for 4 h in RPMI 1640, 5% fetal calf serum containing 100 μg/ml 35S-methionine. In other experiments, cells were pulsed for 10 min in RPMI 1640, 5% fetal calf serum plus [35S]methionine at 500 μCi/ml. The cells were washed in ice-cold medium, resuspended in normal RPMI 1640 with 10% fetal calf serum, and recultured for differing “chase” intervals.

- 32P Labeling—Labeling with 32P was carried out in phosphate-free medium (30 mM Heps, pH 7.4, 110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 2 mM glutamine, 2 mg/ml bovine serum albumin). LK63 cells were resuspended at 4 × 10^6/ml and incubated for 1 h with 32P at 50 μCi/ml. Labeling was quenched by washing twice in ice-cold phosphate-buffered saline.

Preparation of Cell Lysates—Cells were lysed in 1% Triton X-100 in 25 mM Tris-HCl, pH 7.4, with 150 mM NaCl. Leupeptin, antipain, chymostatin, and pepstatin (each at 1 μg/ml) were added to prevent proteolysis. After 45 min on ice the lysates were clarified by ultracentrifugation.

Immunoprecipitations—The lysates were precleared overnight with 50 μl of protein A-Sepharose 4B beads and 10 μg of rabbit anti-mouse IgG antibody. Supernatants were transferred to fresh tubes, mixed with monoclonal antibodies, and held on ice for at least 4 h. The immune complexes were precipitated on rabbit anti-mouse Ig and protein A-Sepharose 4B (as above).

In some cases antigen was absorbed directly to III.A4 antibody conjugated to Trisacryl beads (see below). In this case the beads were mixed with the supernatant and mixed at 4 °C on an end-over-end stirrer for 2 h. In each case the beads were washed twice with 1% Triton X-100, 0.2% deoxycholate, 25 mM Tris, pH 7.4, 0.5 mM NaCl, 1 mM EDTA, 1 mM EGTA and twice with the same buffer except that NaCl was 0.1 M and 0.01% SDS was added. The samples were eluted into running buffer and analyzed by SDS-PAGE.

Glycosylation Studies

Glycosylation Inhibitors—Cells were incubated for 6 h with biosynthetic labeling medium at 5 × 10^6/ml in the presence of 10 μg/ml tunicamycin (Sigma), 2 mM deoxymannojirimycin (Boehringer Mannheim), 100 μM castanospermine (Boehringer Mannheim), or with medium alone. The radiolabeled hek antigen was analyzed as described above.

Endoglycosidase Treatment—In other experiments cells were labeled with 35S methionine. Glycosylation was inhibited with tunicamycin, deoxybenomannose, and treated in different concentrations of endoglycosidase. The samples were subjected to SDS-PAGE and analyzed by autoradiography.

In Vitro Kinase Reactions

Cell lysates of unlabeled LK63 cells were prepared and lysates incubated with III.A4-conjugated Trisacryl beads. Pure III.A4 antibody was isolated in protein A-Sepharose coupled to glutaraldehyde-activated Trisacryl at 2.5 mg of III.A4/ml according to the manufacturer’s protocol (IBF, France). Beads were washed and described above followed by two washes with kinase buffer (50 mM Hepes, pH 7.4, 10 mM MnCl2, 1% Triton X-100). The washed beads were incubated in kinase buffer containing 20 μCi of [γ-32P]ATP for 15 min at room temperature. In some experiments 0.1 μg of dephosphorylated antigen was added as a substrate to these reactions. The reaction was stopped by the addition of an equal volume of 2 × SDS sample buffer. The samples were analyzed on 7.5% SDS-PAGE gels.

Analysis of Phosphomimetic Avidin

Dried slab gels from the in vitro kinase experiments were analyzed with autoradiographs and bands containing 32P-labeled phosphoproteins excised from a gel. The gel slices were rehydrated and washed twice in 20 mM NH4HCO3 (1 h/wash). Protein was eluted by treatment with 100 μl of proteinase K (0.5 mg/ml) in 20 mM NH4HCO3. The eluates were pooled and lyophilized and then treated with 100 μl of 6 N HCl for 1 h at 110 °C. After rehydropolization the

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetracetate acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
material was taken up in 5 μl of pyridine/acetic acid/water at 10:100:1890 ratio (pH 3.5). Samples and phosphonamidase acid standards (tyrosine, serine and threonine, Sigma) were analyzed on precoated cellulose thin layer chromatography plates (Merck 3757) by two-dimensional chromatography performed as described previously (28) using an HTE-2-L chromatography apparatus (C.P. Co., Del Mar, CA). The TLC plates were stained with 1% ninhydrin, dried, and autoradiographed.

### Antigen Purification

LK63 cells were prepared in batches of 10⁶ cells and lysed in 100 ml of lysis buffer (25 mM Tris, pH 7.4, 0.5 M NaCl, 1% Triton X-100, plus 50 μg of sodium dodecyl sulfate) as described previously. The lysate was centrifuged at 4,000 × g to remove unlysed nuclei. The supernatant was centrifuged at 35,000 × g in a Sorvall RC4B centrifuge for 1 h at 4 °C. The lysate was incubated with Sepharose 4B beads (2% v/v) overnight. The absorbed lysate was incubated with Trisacryl beads coupled with FMC63 (CD19) antibody for >4 h. The FMC63-absorbed lysate was incubated with 2 ml of III.A4-coupled Trisacryl beads for 4 h. The slurry was transferred to a small column and the lysate was removed. The III.A4 beads were washed with 20 volumes of lysis buffer and with 10 volumes of 10 mM Tris, pH 7.4, 0.5% Triton X-100. The beads were washed sequentially with 4 volumes of 50 mM ethylene diamine diethylamine (pH 11) and 0.1% Triton X-100 and 100 volumes of 125 mM Tris-HCl buffer, pH 6.8, containing 20% glycerol, 1 mM EDTA, 50 mM acrylamide gel. Gel slices were overlayed with 50 μl of 125 mM polyacrylamide gel, pyridylethylated the following protocol. The lyophilized eluate was dissolved in 125 mM Tris-HCl buffer, pH 8.1, containing 15% (v/v) methanol and 1 mM thioglycollic acid/H₂O (12:7:81). Protein bands were excised, soaked for 1 h in 10% SDS-PAGE. The gels were stained with a modified silver stain protocol (Bio-Rad). The III.A4 bead was washed with 20 volumes of lysis buffer and with 10 volumes of 10 mM Tris, pH 7.4, 0.5% Triton X-100. The beads were then eluted sequentially with pH 11, 12, and 12.5% 50 mM diethanolamine, 0.1% Triton X-100. Samples were removed from each eluate, neutralized with HCl, lyophilized, and analyzed by 7.5% SDS-PAGE. The gels were stained with a modified silver stain protocol (Bio-Rad). The hek protein eluted at pH 11-12. These fractions were lyophilized and lyophilized for sequence analysis.

### Amino Acid Sequence Analysis

**Electrophoresis and Electrotransfer**—The N-terminal sequence of hek was determined as described previously (29). Briefly, hek was separated using a 7% polyacrylamide gel employing the Laemmli buffer system and the Bio-Rad protein II apparatus. The separating gel was preelectrophoresed at 60 V for 4 h in 375 mM Tris-HCl buffer, pH 8.8, containing 1 mM thiglycolllic acid prior to pouring the stacking gel. Electrophoresis was performed at 35 mA constant current, the temperature being maintained at 4 °C by circulation of water from an external cooling system. Thiglycolllic acid (1 mM) was included in the upper buffer reservoir during electrophoresis. After electrophoresis, the gel was equilibrated in 10 mM CAPS buffer, pH 11, containing 15% (v/v) methanol and 1 mM thiglycolllic acid for 30 min prior to electroblotting onto polyvinylidene difluoride membrane. The transfer was performed at 30 V for 2 h at 4 °C using a Bio-Rad Transblot apparatus. Protein was stained using 0.1% Coomassie Blue in 50% methanol and destained using 50% methanol, 10% acetic acid and then washed extensively with distilled water to remove contaminating acids which otherwise interfere with interpretation of the initial Edman degradation cycles (29).

**Peptide Mapping: In Situ Proteolytic Digestion of Proteins in Acrylamide Gel Matrix**—The eluate of the III.A4-Trisacryl column was removed. The III.A4 beads were washed with 20 volumes of lysis buffer and with 10 volumes of 10 mM Tris, pH 7.4, 0.5% Triton X-100. The beads were then eluted sequentially with pH 11, 12, and 12.5% 50 mM diethanolamine, 0.1% Triton X-100. Samples were removed from each eluate, neutralized with HCl, lyophilized, and analyzed by 7.5% SDS-PAGE. The gels were stained with a modified silver stain protocol (Bio-Rad). The hek protein eluted at pH 11-12. These fractions were lyophilized and lyophilized for sequence analysis.

**Antigen Purification**

### RESULTS

**Phenotypic Analysis of hek Antigen Expression Using the III.A4 Antibody**—The expression of the hek antigen detected by the III.A4 monoclonal antibody was initially analyzed on human cell lines. The expression of the antigen on the immunizing cell line (LK63) is depicted in Fig. 1. It is evident that III.A4 antibody detected significant levels of its target hek antigen on this cell line but failed to detect the antigen on two other hemopoietic cell lines. Analysis of the extensive group of lines depicted in Table I revealed only one other III.A4-positive cell line (JM). The Raji cell line showed weak expression, but all other lines tested were consistently negative. In particular, four other pre-B cell lines showed no detectable expression.

Normal and tumor tissue isolated from patients undergoing biopsy procedures allowed us to analyze expression in vivo. As shown in Table II, hek was not detectable on normal tissues by the methods employed. Analysis of tumors showed that a very low proportion of hemopoietic tumors expressed the hek antigen. Moreover, analysis of early passage LK63 cells showed that hek antigen was detectable within a few passages of initial in vitro culture, suggesting that elevated hek antigen expression on the LK63 tumor might be an in vivo event. However, no direct ex vivo tumor tissue could be examined, this could not be confirmed.

**Molecular Properties of the hek Antigen**—The results of phenotypic analysis suggested that the hek antigen was expressed at an abnormally high level on LK63, perhaps indicating a role for this antigen in the oncogenic process. We used the III.A4 antibody to analyze the molecular properties of the hek antigen. As shown in Fig. 2, cell surface labeling with fluorescein isothiocyanate-conjugated III.A4 antibody revealed a protein of 135 kDa. To determine the glycosylation status of the protein two experiments were performed. The first was a biosynthetic labeling of LK63 cells treated with inhibitors of glycosylation. Tunicamycin was extremely toxic to the cells, but a faint band was detected at 95 kDa (Fig. 3). Castanospermine and deoxyxymonojirimycin treatments resulted in intermediate sized glyco-
TABLE II
Detection of hek on freshly isolated human tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Method of analysis</th>
<th>Flow cytometry</th>
<th>IMPOX</th>
</tr>
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<tbody>
<tr>
<td>Normal spleen</td>
<td></td>
<td>0/3</td>
<td>0/2</td>
</tr>
<tr>
<td>Normal lymph node</td>
<td></td>
<td>0/4</td>
<td>0/5</td>
</tr>
<tr>
<td>Normal bone marrow</td>
<td></td>
<td>0/6</td>
<td>NT*</td>
</tr>
<tr>
<td>Normal tonsil</td>
<td></td>
<td>0/2</td>
<td>NT</td>
</tr>
<tr>
<td>Normal breast</td>
<td></td>
<td>NT</td>
<td>0/3</td>
</tr>
<tr>
<td>Normal brain</td>
<td></td>
<td>NT</td>
<td>0/2</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td></td>
<td>1/28</td>
<td>NT</td>
</tr>
<tr>
<td>Lymphoma</td>
<td></td>
<td>0/85</td>
<td>0/9</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td></td>
<td>2/39</td>
<td>NT</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td></td>
<td>0/39</td>
<td>NT</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td></td>
<td>NT</td>
<td>0/8</td>
</tr>
<tr>
<td>Other carcinomas</td>
<td></td>
<td>NT</td>
<td>1/6</td>
</tr>
</tbody>
</table>

* Primary hematopoietic tumors and tissues were prepared as single cell suspensions and stained by indirect immunofluorescence.
* Tissue sections were analyzed by the immunoperoxidase (IMPOX) technique.
* NT = not tested.
* Specimens included one cervical, one prostate, one ovarian, and one renal carcinoma (positive) and two carcinomas of unknown origin.

FIG. 2. Immunoprecipitation of hek antigen by III.A4 antibody. A, autoradiograph of immunoprecipitates as from 125I-labeled LK63 cell lysate. Lane 1, immunoprecipitation with III.A4-Trisacryl beads. Lane 2, immunoprecipitation with control antibody (LA7 anti-CD36) coupled to Trisacryl beads. B, silver staining of immunoprecipitation of LK63 cell lysate. Lane 1, elution at pH 11 from III.A4-Trisacryl beads. Lane 2, pH 11 eluate of immunoprecipitate of run-through cell lysate on II.A4-Trisacryl. Lane 3, eluate of LA7-Trisacryl column.

FIG. 3. Biosynthetic labeling of hek antigen in the presence of inhibitors of glycosylation. [35S]Methionine labeling was performed in the presence of tunicamycin (TUN), deoxymannojirimycin (DMN), castanospermine (CSP), and medium alone. The lysates were immunoprecipitated with III.A4-Trisacryl beads and the samples analyzed by SDS-PAGE. The arrow indicates the faint band seen after labeling in the presence of tunicamycin.

FIG. 4. Deglycosylation of hek antigen by N- and O-glycanase. LK63 cells were labeled with 125I and the cell lysate immunoprecipitated with III.A4. Shown are a control precipitate (−) and a precipitate treated sequentially with N- and O-glycanase (+ ENZ).

FIG. 5. 32P labeling of LK63 cells. Autoradiograph of immunoprecipitation of LK63 cells using A, III.A4-Trisacyl beads; B, B1(CD20) antibody bound to protein A-Sepharose 4B.

FIG. 6. hek antigen is labeled in an in vitro kinase reaction. Immunoprecipitates of hek and surface IgM were prepared using III.A4-Trisacryl and S.ADA.14 (anti-p chain) antibodies from unlabeled LK63 (A, B, E, and F) or 125I-labeled cells. In vitro kinase reactions were analyzed. Lanes A and E, two exposures of the precipitates with III.A4. Lanes C and D show, respectively, parallel autoradiographs of 125I-labeled hek (lane C) and p chain (lane D).

hek, a Novel Human Receptor-type Protein Tyrosine Kinase

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inconstantly seen and presumably represent contaminating phosphoproteins present as a result of incomplete removal of other cellular proteins. The major band was excised and subjected to phosphamino acid analysis which confirmed phosphorylation on tyrosine (Fig. 7). In this case, dephosphorylated casein was added to the reaction mixture. It is evident that thehek and casein bands are heavily phosphorylated. The phosphamino acid analysis of each band showed phosphorylation on tyrosine.

Isolation of the Antigen Detected by III.A4—To confirm that thehek protein was indeed a tyrosine kinase we sought amino acid sequence information. The protein was purified by scaling up the isolation procedure using III.A4 antibody coupled to Trisacryl. Other affinity supports failed to give clean purification, but this support yielded hek protein in high purity as analyzed by silver staining of SDS-PAGE gels (Fig. 2B). Ten-liter batches of cells (about 10^9 cells) were prepared for antigen isolation. Each batch yielded 5–10 μg (55–110 pmol) of purified hek antigen based on comparison with protein standards. Initially, N-terminal sequencing was performed on two separate batches of material. In each case the same sequence was detected (Table III). Internal sequence analysis was obtained by in situ digestion of hek protein (10 μg, 111 pmol) in the polyacrylamide matrix with staphylococcal V8 protease. The generated fragments were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. The peptide map obtained, after visualization by Coomassie Blue R-250 staining, is presented in Fig. 8. Peptides 1 and 2 (Fig. 8) were subjected to N-terminal sequence analysis and were found to have identical N-terminal sequences (Table III). Other bands were also sequenced but did not yield sequence data amenable for subsequent gene cloning studies.

![Fig. 8. SDS-PAGE of staphylococcal V8 protease digest of hek protein.](image)

DISCUSSION

We have shown that a monoclonal antibody raised against the human LK63 pre-B cell line (Fig. 1) is specific for a 135-kDa glycoprotein designated hek (Figs. 2 and 3). This protein was clearly expressed by LK63 cells and by a T cell line JM (Table I). However, many other cell lines and a number of normal tissues did not stain with this antibody by immunofluorescence and/or immunoperoxidase techniques (Tables I and II). When fresh tumor specimens were examined, a small number of cases showed detectable hek expression (Table II). These findings suggested sporadic overexpression of hek on human tumors. We postulated that hek may be an oncogetic protein and, given its cell membrane localization, we explored the possibility that hek is a tyrosine kinase. We showed that hek was only weakly phosphorylated by biosynthetic labeling with ^32P (Fig. 5). Moreover, we were unable to increase the level of phosphorylation by exposure to a number of known growth factors and chemical inducers (data not shown). This level of labeling was insufficient to allow a direct demonstration of tyrosine phosphorylation in these experiments. However, in vitro kinase reactions performed with purified hek protein and a kinase substrate (casein) allowed us to show that hek was autophosphorylated on tyrosine and could tyrosine phosphorylate casein (Fig. 7). To exclude the possibility that the kinase activity was generated by a minor, copurifying protein, sequence data were obtained (Table III). The sequence of V8 protease peptides (Fig. 8) confirmed that hek is a member of the tyrosine kinase family.

Several known tyrosine kinases have a molecular weight similar to hek. However, the N-terminal sequence obtained (Table III) is unique according to data base searches (EMBL, Swissprot, GenBank, Japanese Protein). However, one internal sequence obtained (peptides P1 and P2) exhibited significant sequence identity to a region of the kinase domain of other protein tyrosine kinases (Table IV). The closest matches are with elk and eph, which are members of the eph family of protein tyrosine kinase (22, 23, 35). Another member of this family, eck (24), is somewhat more divergent. Partial sequences of eck and erk (36) show that hek is also quite different from eck. The relevant amino acid sequences are not published for erk, but preliminary polymerase chain reaction-derived nucleotide sequences of hek (data not shown) show that it is also distinct from erk. Comparison with other receptor kinases shows marked divergence over this region of sequence, strongly suggesting that hek is a member of the eph family.

**FIG. 7. Phosphamino acid analysis of hek antigen and casein after an in vitro kinase reaction of proteins immunoprecipitated with III.A4 antibody.** Immunoprecipitated hek antigen bound to III.A4- Trisacryl was mixed with casein in a kinase reaction (as above). After SDS-PAGE the reaction labeled hek (p135) and casein were identified (lane 1). The bands were removed and subjected to phosphamino acid analysis (see "Materials and Methods").

**TABLE III**

<table>
<thead>
<tr>
<th>Sequence obtained from purified III.A4 antigen</th>
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<tr>
<td>The sequences obtained were confirmed on two independent specimens. The internal sequence was obtained from peptides P1 and P2 (Fig. 8). The yields of phenylthiohydantoin derivative observed in the first cycle of the Edman degradation for each sequence are given in parentheses.</td>
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<table>
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<tr>
<td>E L I P Q P S N E V N L D X (S) K X I Q (26 pmol)</td>
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<table>
<thead>
<tr>
<th>Internal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>G Y R L P P P M D C P A L Y Q L M L D C (10 pmol)</td>
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subfamily of protein tyrosine kinase. The closest similarity is with ekl, a rat tyrosine kinase that has been shown to have restricted expression to brain and testis (23, 37). Whether hek is the human homologue of ekl or a different but closely related member of the eph family remains to be determined once more extensive sequence data are available. However, it is notable that the N-terminal sequence of rat ekl (37) is quite unlike the N-terminal sequence of hek. Moreover, the other internal sequences, although limited, appear not to have a counterpart in the ekl sequence or indeed with the sequences of eph or eck (data not shown). Further comparison with known eph-like kinases requires the molecular cloning of hek which will allow detailed sequence comparison.

The overexpression of hek on some hemopoietic tumors suggested a role in tumorigenesis. We have performed Scatchard analysis with [125I]labeled III.A4 and demonstrated that both LK63 and JM have 10,000-20,000 hek sites/cell. In contrast, no detectable sites were found on Raji or K562 cells (data not shown). This would appear to imply that hek is not expressed at all on some related hemopoietic tumor cell lines. One possibility is that hek is aberrantly expressed rather than overexpressed on these lines.

In conclusion, this study has identified a novel human receptor-type tyrosine kinase. The hyperexpression of hek in a number of tumors suggests that this protein may play a role in tumor induction. HER/neu/her-B2 has been shown to be expressed aberrantly on some breast and oesophageal carcinoma (38). Moreover, amplified expression of neu correlates with bad prognosis in human breast cancer (39). Another protein tyrosine kinase, ERBB3, was also shown to be overexpressed in a subset of human breast tumors (40). Within the eph family, the eph protein itself has already been shown to be expressed in a number of colon carcinomas (22, 41). The present findings suggest that hek, another member of the eph family, also has oncogenic properties.

REFERENCES