Overexpression of ERK, an EPH Family Receptor Protein Tyrosine Kinase, in Various Human Tumors¹

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Abstract

The ERK gene has been isolated as a genomic DNA encoding a part of the receptor protein-tyrosine kinase which belongs to the EPH subfamily. We previously identified a partial complementary DNA (cDNA) encompassing the catalytic domain of ERK from the expression library of human gastric cancer with an antiphosphotyrosine antibody. Using this cDNA as a probe, the cDNAs encoding mature ERK protein were isolated. The putative mature ERK protein, a total of 967 deduced amino acid residues, showed high homology with chicken Cek5 (92.5%) and mouse Nuk (99.1%). Chromosomal in situ hybridization revealed that human ERK cDNA is localized to chromosome 1p34–35. In Northern blot analysis of normal human tissues, the ERK gene was ubiquitously expressed mainly in levels in various tumors of epithelial origin than in corresponding normal tissues, most frequently in gastric cancers (12 of 16, 75.0%). Overexpression of ERK was also detected in one osteosarcoma cell line. These findings suggest that ERK plays some significant role in carcinogenesis in the stomach and other tissues.

Introduction

Protein-tyrosine kinases are classified into two types according to their primary structure based on deduced amino acid sequences. One is the nonreceptor type, and the other is the receptor type consisting of nine subfamilies (1). Some of them are reported to be structurally and functionally related enzymes intimately involved in signal transduction associated with cell growth and carcinogenesis (1). The EPH subfamily genes encoding receptor protein-tyrosine kinases are structurally characterized by the juxtaposition of a vestigial immunoglobulin-like (IgL) domain, a single cysteine-rich region, and two FN III domains in the extracellular region (2, 3). Thus far, at least 11 fully sequenced members have been reported in the EPH subfamily, including EPH (2), ECK (4), HEK (5), HEK 2 (6), Elk (7), Ekh-1, Ekh-2 (8), Sek (9), Cek5 (10) and Cek4/Mek4 (11). In addition to these, a few other members have been partially sequenced (12–15). We previously isolated a partial ERK cDNA covering the catalytic domain and the COOH-terminal tail from a cDNA library of a well-differentiated gastric adenocarcinoma tissue and reported that expression of ERK was developmentally regulated in the rat stomach and highly elevated in certain human gastric cancer tissues (15). Here we report the isolation of the cDNA sequence covering the coding region of mature ERK protein, chromosomal mapping by in situ hybridization, and overexpression in various human tumors.

Materials and Methods

Tissues and Cell Lines. Samples of human tumor and normal tissues were obtained at the time of surgical removal and at autopsy. The tumor tissues studied were taken from 16 gastric cancers, 11 colon cancers, 6 esophageal cancers, 7 ovarian cancers, 2 renal cell carcinomas, 1 lung cancer, 1 malignant thymoma, and 1 lymphoma. Human gastric cancer cell line NKPS was a gift from Dr. M. Mai, Cancer Research Institute of Kanazawa University, Kanazawa, Japan. TMK 1 was a gift from Dr. W. Yasui, the First Department of Pathology of Hiroshima University, Hiroshima, Japan. CT nu-1 was derived from malignant carcinoid of the duodenum and established in our department. The other human cell lines were purchased from the Japanese Cancer Research Resource Bank. All of the samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA preparation.

RNA Preparation. Total RNAs were extracted from the various human tissues and cell lines by guanidinium/cesium chloride ultracentrifugation (16). Poly(A)+ RNA was obtained by oligo(dT) cellulose column chromatography (16).

Construction and Screening of cDNA Libraries. The cDNA libraries were synthesized from 5 μg of poly(A)+ RNA obtained from human gastric cancer cell line MKN 28 by the cDNA synthesis system plus using an oligo(dT) primer or a random primer (Amersham, Buckinghamshire, United Kingdom). The cDNAs were filled in with T4 DNA polymerase and ligated to the adaptor containing an EcoRI site (Takara, Kyoto, Japan). The cDNA libraries were ligated to EcoRI-digested Agt10 under conditions recommended by the supplier (Stratagene, La Jolla, CA). Ligated DNAs were then packaged into phage particles with Gigapack II Gold Packaging Extract (Stratagene). Filters prepared from the cDNA libraries were hybridized with [α-32P]dCTP-labeled probes and washed under highly stringent conditions according to the method of Sambrook et al. (16).

DNA Sequencing. The cDNA inserts isolated from the libraries were subcloned into the pBluescript KS+ plasmid vector, and sequenced by the double-strand dyeoxy-chain termination method using T7 DNA polymerase (Sequenase Version 2.0; United States Biochemical, Cleveland, OH). The sequences were determined using universal primers and synthetic primers complementary to each cDNA and analyzed with the program GENETYX (Software Development Co., Tokyo, Japan). A homology search program, FASTA, was performed in the GenBank and EMBL nucleic acid databases using an on-line system to DDBJ (Mishima, Japan).

Northern Blot Analysis. Total RNAs from human tissues and cell lines were separated on 1.0% agarose gel containing formaldehyde and transferred to nitrocellulose filters (NitroPlus; Micron Separation Inc.). The probe was labeled with [α-32P]dCTP using a random primer DNA labeling kit (Takara). Hybridization and washing conditions were as described by Sambrook et al. (16).

Chromosomal in Situ Hybridization. R-bandened chromosomes were prepared from human lymphocytes following the methods of Viegas-Pequignot and Dutrillaux (23) as modified by Takahashi et al. (24). The 2.6-kilobase cDNA fragment covering exons of ERK cloned into the pGEM-T plasmid vector (Promega, Madison, WI) was used as a probe. The probe was labeled by nick translation with biotin-16-dUTP. The standard FISH methods developed

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3 The abbreviations used are: FN III, fibronectin III; cDNA, complementary DNA; FISH, fluorescence in situ hybridization; poly(A)+ RNA, polyadenylated RNA; oligo(dT), oligo(dT)cellulose chromatography.
**Fig. 2. Expression of ERK in normal human tissues.** Lane 1, cerebellum; Lane 2, cerebrum; Lane 3, thyroid; Lane 4, kidney; Lane 5, lung; Lane 6, adrenal; Lane 7, colon; Lane 8, liver; Lane 9, stomach; Lane 10, ovary; Lane 11, testis; Lane 12, epidermidis; Lane 13, submandibular gland; Lane 14, heart; Lane 15, spleen; Lane 16, prostate. Samples of total RNAs, 10 μg each, were hybridized with α-32P-labeled MSO.4. Hybridization of the same filters with β-actin probe shows the quality and quantity of the RNAs. Transcript sizes are indicated in kilobases (kb). The autoradiographs were obtained after overnight exposure at —80°C.

by Lawrence et al. (25) were modified by Takahashi et al. (26) and Takai et al. (17). The signal amplification procedure was carried out by the methods described previously (18). The slides were finally stained with propidium iodide, and the chromosomes were examined using a Nikon OPTIPHOT-2-EFD2 microscope (B-2A filter). A B-2E filter was used to detect only FISH signals.

**Results**

**Isolation of cDNA Clones and Nucleotide Sequence Analysis.** To isolate larger cDNAs of ERK, the cDNA library was constructed from the poly(A)^+ RNA of MKN28, a gastric cancer cell line in which ERK is highly expressed. A total of 2.4 × 10^6 recombinant phages of the oligo(dT)-primed cDNA library were screened with the clone H1, a previously described 1.2-kilobase fragment of ERK cDNA, as a probe (Fig. 1a) (15). Among the seven positive clones isolated, the one with largest insert, containing 2.6 kilobases, was designated λERK5, 0.3 kilobase of which covered the 3'-untranslated region, and the remaining 2.3 kilobases were from the coding region of the cDNA. To obtain sequences upstream to this cDNA, a 1.0-kilobase cDNA fragment located at the 5' end of λERK5 was amplified by polymerase chain reaction using the universal primer and a synthetic primer complementary to the cDNA. Fourteen overlapping clones were isolated after screening 4.0 × 10^9 phages of the random clone Hi, a previously described 1.2-kilobase fragment of ERK cDNA, as a probe (Fig. 1a) (15). At least two of these, referred to as λERK6 and λERK7, contained an additional 0.7- or 0.8-kilobase sequence to the 5' terminal (Fig. 1b). The composite nucleotide sequence of human ERK cDNA, compiled from the four overlapping clones, spans 3118 base pairs (Fig. 1b). The cDNA sequence contains an open reading frame of 2934 base pairs encoding 978 amino acid residues. From the comparison of amino acid sequences of other EPH subfamily members, ERK cDNA contained a small, incomplete signal peptide region at the amino terminal (7, 10). Because the consensus cleavage site of a signal sequence was located between residues 11 and 12 (19), we conclude that the cDNA located at the nucleotide 206—639 in human ERK cDNA, contained a small, incomplete signal peptide region at the amino terminal (7, 10). Because the consensus cleavage site of a signal sequence was located between residues 11 and 12 (19), we conclude that the cDNA located at the nucleotide 206—639 in human ERK cDNA, contained a small, incomplete signal peptide region at the amino terminal (7, 10).

The expression level of ERK mRNA in various organs of fetal and adult rats was the same method. ERK was highly expressed in the whole brain of fetal day 16 rats, but not in adults (data not shown).

**Expression of ERK in Tumors.** Northern blot analysis of 31 human tumor cell lines using the MSO.4 probe revealed that ERK is expressed at various levels in several cell lines of epithelial origin. Among them, high level expression was detected in NKPS, KATO III, MKN28 (gastric cancer), TE 1 and 10 (esophageal cancer), COLO 201 (colon cancer), KPK (renal cell carcinoma), PA-1 (teratocarcinoma), and BeWo (choriocarcinoma). In contrast, among tumors of nonepithelial origin, the expression level of ERK was high only in osteosarcoma, Huo-3N1, and very low in promyelocytic leukemia cell line HL60 (Fig. 3a), while a member of the EPH family, HEK, showed specific expression in lymphoid tumor cell lines (5). To determine if human ERK gene expression is altered in neoplastic tissues, we analyzed RNAs from 45 human tumors and corresponding normal tissue by Northern blot hybridization. The amount of ERK mRNA was at least several times higher with a major transcript size of 4.2 kilobases and a minor transcript of 11.0 kilobases, in the various common human carcinomas than in normal tissue, including carcinoma of the stomach (12 of 16 (Fig. 3b), 8 typical cases), colon (10 of 11 (Fig. 3c), 5 typical cases), esophagus (3 of 6), ovary (1 of 7), kidney (1 of 2), and lung (1 of 1). The expression of ERK was not detected in tissues of one lymphoma or one malignant thymoma (data not shown). Although there was no particular preference for any histological type of cancer, the prevalence of ERK overexpression seemed to differ in two subtypes of gastric cancer. As shown in Table 1, overexpression of ERK was detected in eight (88.9%) of nine cases of well-differentiated adenocarcinoma and in four (57.1%) of seven cases of poorly differentiated adenocarcinoma. Elevated levels of ERK mRNA in gastric cancers were detected at an early stage [three of three (Fig. 3b, Case 1—3)]. In most cases of advanced colon cancer that we examined, on the other hand, ERK mRNA was expressed almost two times homologous to FN III (residues 315—383 and 434—521), which are characterized by the presence of conserved aromatic and hydrophobic residues (10).
Fig. 3. Expression of ERK in various human tumors. Samples of total RNAs 10 μg each, were hybridized with α-32P-labeled MS0.4. a, examples of 31 cell lines. Lanes 1–5, gastric cancer; Lanes 6–12, esophageal cancer; Lanes 13 and 14, colon cancer; Lanes 15, renal cell carcinoma; Lanes 16, teratocarcinoma; Lanes 17, choriocarcinoma; Lane 18, carcinoid; Lanes 19–23, osteosarcoma; Lanes 24, hepatoblastoma; Lanes 26–31, leukemia. b, examples of eight typical cases of gastric cancer. Numbers above the lanes, case numbers; T, "tumor tissue"; N, corresponding "normal tissue." Cases 1–4, well-differentiated adenocarcinoma (papillary); Case 5, well-differentiated adenocarcinoma (papillotubular); Cases 6 and 7, poorly differentiated adenocarcinoma (tubular); Case 8, poorly differentiated adenocarcinoma (solid); Cases 1–3, early stage carcinomas; Cases 4–8, advanced stage carcinomas. c, examples of five typical cases of colon cancer. All cases are advanced stage adenocarcinomas. Autoradiographs were obtained after overnight exposure at —80°C. Hybridization of the same filters with β-actin probe is shown as a control. Transcript sizes are indicated in kilobases (kb).

Table 1 Expression of ERK mRNA in 16 histologically examined gastric cancers

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<td>Well differentiated</td>
<td>5/6</td>
<td>3/3</td>
<td>8/9</td>
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<tr>
<td>Poorly differentiated</td>
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a Number of tumors overexpressing ERK/number tested.
b Poorly differentiated includes papillary and papillotubular adenocarcinoma. Poorly differentiated includes tubular adenocarcinoma and signet ring cell carcinoma.
c Early cancers include carcinomas confined to the mucosa and the submucosa.

at the same level in corresponding normal mucosa (Fig. 3c). There was no amplification or gross rearrangement of ERK by Southern blot hybridization with the whole λERK6 cDNA probe in cases of the gastric cancer with overexpression (data not shown).

Chromosomal in Situ Hybridization. Among 50 metaphase cells hybridized with the probe, 36 cells displayed symmetrical double spots on at least one copy of the short arm of chromosome 1 at band p34–35 (Fig. 4). No paired signals were detected on any other chromosome. On the basis of this finding, we mapped that the ERK
gene to the human chromosomal region 1p34–35 using FISH with R-banded chromosomes directly.

Discussion

The sequence of ERK cDNA we determined is closely related to Cek5 and Nuk and belongs to the EPH subfamily of receptor-tyrosine kinase. Although the similarity of the signal peptide region is not clear, ERK appeared to be the human counterpart of Cek5 and Nuk. Within the EPH subfamily, ERK, like Cek5, Sek, Eth-1 and -2, and the Cek4/Mek4/HEK, has greater similarity to Elk than to the ECK or EPH proteins. At present, no extracellular ligands for any members of EPH subfamily have been identical, and hence, little is known about the biological functions of these receptor-tyrosine kinases. The presence of FN III repeats and the IgL domain may account for the possible involvement of this gene in cell adhesion processes (3).

According to our results, ERK mRNA is expressed ubiquitously in various human organs of epithelial origin, although the genomic DNA fragment of ERK has been reported to be expressed most abundantly in the lung of the adult mouse (12). Many other members of the EPH subfamily are notably or chiefly expressed in the nervous system (7–12). Since the human brain tissues that we examined were randomly sampled from the frontal cortex and cerebellar cortex, there is the possibility that ERK is expressed in certain restricted regions of the brain. Our results in the rat also suggest that ERK may have a function in the brain during embryonic development.

Partial ERK was reported to be located to chromosome 1 (12), and we detected its precise location on 1p34–35. Although various 1p deletions, especially 1p35 and 36, have been identified in some colorectal adenomas and carcinomas (21, 22), it remains uncertain whether these are related to ERK expression.

Here we show that the newly isolated receptor-tyrosine kinase ERK is overexpressed in common human malignancies, including human gastric cancer. Interestingly, ERK expression was noted especially in gastric cancer tissues with papillary formations, although in the cell lines there was no correlation between ERK expression and their original histological types. Considering the heterogeneity of the histopathological features and biological behavior of human gastric cancer, it is intriguing to speculate that ERK is involved in the carcinogenesis of certain subtypes of human gastric cancer at an early stage. The significance of overexpression and its mechanistic basis require further investigations, including detailed characterization of ERK cDNA and in situ localization of its gene product.

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cDNA CLONING OF ERK


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