Preferential Expression of the Third Immunoglobulin-like Domain of K-sam Product Provides Keratinocyte Growth Factor-dependent Growth in Carcinoma Cell Lines

Hideshi Ishii, Yutaka Hattori, Hiroshi Itoh, Tatsuya Kishi, Teruhiko Yoshida, Hiromi Sakamoto, Hakumei Oh, Sho Yoshida, Takashi Sugimura, and Masaaki Terada

ABSTRACT

Previously, we identified an amplified gene in a stomach cancer cell line, KATO-III, and designated it K-sam. This gene was later found to be identical with a gene for a receptor tyrosine kinase, bek/FGFR2. One of the characteristics of the K-sam gene is structural diversity of its transcripts; K-sam complementary DNA (cDNA) cloned from human brain (K-sam-I) has a completely different sequence at the third extracellular immunoglobulin-like domain as compared to that of the K-sam cDNA derived from KATO-III cells (K-sam-II). Recent study has revealed that this difference signifies a differential ligand affinity; the receptor encoded by the K-sam-I cDNA has a high affinity for basic fibroblast growth factor (bFGF), while the K-sam-II cDNA corresponds to a receptor with the high affinity for keratinocyte growth factor (KGF). Reverse transcription-polymerase chain reaction and RNA blot analysis showed that the K-sam-II type transcript was present in carcinoma cell lines but not in any of the sarcoma cell lines examined. The K-sam-I type transcript was expressed in both carcinoma and sarcoma cell lines. Furthermore, KGF enhanced the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial. In contrast, the DNA synthesis of the esophageal cancer cells, TE-1, in a dose-dependent manner, while the effect of bFGF was not substantial.

INTRODUCTION

We previously reported a receptor tyrosine kinase gene, K-sam, which was isolated as an amplified gene in a human stomach cancer cell line, KATO-III, by the in-gel DNA renaturation method (1–3). The K-sam-related genes, N-sam and sam3, have been identified (4, 5). An analysis of the nucleotide sequences of the K-sam, N-sam, and sam3 cDNAs revealed that they are members of the FGFR gene family (4–6). At least four distinct FGFRs, that is, FGFR1, FGFR2, FGFR3, and FGFR4, have been isolated to date (7–10); K-sam is identical to the human bek/FGFR2 gene (6), N-sam is identical to human FLG/FGFR1 (4), and sam3 is presumably the rodent counterpart of human FGFR3 (5).

We have identified at least four types of K-sam cDNA from various sources (11). K-sam-I and K-sam-II cDNAs encode membrane-bound receptors, whereas K-sam-III and K-sam-IV appear to represent secretory forms of the K-sam receptors. K-sam-I cDNA was cloned from normal human brain cDNA library, and K-sam-II cDNA was from the KATO-III cDNA library (11). Comparison of the K-sam-I and K-sam-II cDNAs revealed completely different nucleotide sequences in the second half of the third extracellular Ig-like domain; this region of the K-sam-II cDNA was identical to that of the KGF receptor, which showed high-affinity binding to KGF but not to bFGF, whereas the corresponding region of the K-sam-I cDNA was identical to that of human bek, which is the high-affinity receptor for bFGF but not for KGF (8, 12–16). A genomic analysis around the third Ig-like domain of K-sam/bek suggests that the K-sam-II type and K-sam-I type messages are transcribed from the same gene by an alternative splicing mechanism (14, 15, 17). We and others have also noted that there are several other splicing variations for the K-sam-I type and K-sam-II type transcripts, including those with and without the first Ig-like domain.

Here we report that the K-sam-II type transcript was expressed only in human cancer cells of epithelial origin, or carcinoma cells, while none of five cancer cells of nonepithelial origin, or sarcoma cells, expressed the K-sam-II transcript. We further showed the stimulation of [3H]thymidine uptake by KGF, but not by bFGF, in a carcinoma cell line containing the K-sam-II type transcript. In contrast, [3H]thymidine uptake of a sarcoma cell line expressing the K-sam-I type transcript was stimulated by bFGF but not by KGF.

MATERIALS AND METHODS

Cells, Culture Conditions, and RNA Preparation. Thirteen cancer cell lines, five sarcoma cell lines, an immature teratoma cell line, and a TE-10 esophageal cancer cell line were used in the present studies (Table 1). KATO-III, HSC-39, MKN45, Lu-143, Lu-140, Lu-135, PC-13, PC-10, PC-3, PC-1, A549, and NCC-IT cells were maintained in RPMI 1640 supplemented with 10% FCS, while TE-1, TE-10, A-172, HT-1080, RD, G-361, and G-402 cells were cultured in RPMI 1640 with 7% FCS, RPMI 1640 with 7% FCS, Dulbecco’s modified Eagle’s medium with 10% FCS, Eagle’s minimum essential medium with 10% fetal bovine serum, Eagle’s minimum essential medium with 10% FCS, McCoy’s medium with 10% fetal bovine serum, and McCoy’s medium with 10% FCS, respectively. Extraction of total RNA from these cultured cells was performed as described elsewhere (18). Polyadenylated RNAs from the human fetal brain and the adult brain were purchased from Clontech (Palo Alto, CA).

RT-PCR. cDNA was synthesized from 1 µg of total RNAs or 0.2 µg of polyadenylated RNAs using M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD). We designed the following three pairs of primers (Fig. 1): upstream primer K7–7, 5’-CAGATCCAGGATATGTTGACGTTGC-3’; and downstream primer K7–11, 5’-CTCACTGATTTTTCCTTTTG-3’ for the K-sam-II type second half of the third Ig-like domain; upstream primer PK-9, 5’-AGATGAGGTGAAGACTGCTGGA-3’, and downstream primer PK-7, 5’-TTTCTCCGACCAGGCTGGGT-3’, for the K-sam-I type transcript; upstream primer NS-10, 5’-CAGATCTTGTTAAGACCTGCGGA-3’; and downstream primer NS-13, 5’-GCTACATGAGGCTGCCCACTG-3’ for the N-sam-type transcript. PCR was carried out for 30 cycles. The thermal cycle conditions were: denaturation at 94°C for 30 s; annealing at 65°C for the K-sam-II type transcript (Tm = 71°C), 55°C for the K-sam-I type transcript (Tm = 61°C), or 62°C for the N-sam-type transcript (Tm = 65°C) for every 30 s; and extension at 72°C for 1 min. The products were separated by electrophoresis in a 3% agarose gel. The nucleotide sequence of the K-sam-II
RESULTS

RT-PCR Analysis of the Expression of the Second Half of the Third Ig-like Domain of the K-sam Transcript. Using the RT-PCR technique, we examined the expression pattern of the second half of the third Ig-like domain of the K-sam-II-type and K-sam-I-type transcripts in human cancer cell lines. The second half of the third Ig-like domain of the K-sam-I cDNA is 80% homologous to the N-sam/FGFR1 cDNA at the nucleotide level (4, 11). In order to avoid a cross-reaction between the K-sam-I and the N-sam sequences in PCR analysis, we designed the downstream primers in the juxtamembrane region and determined the respective annealing temperatures by deducing $T_{m}$s (Fig. 1). We ascertained by PCR analysis that the three pairs of primers for the K-sam-II, K-sam-I, and N-sam cDNAs amplify only the corresponding sequences (Fig. 2A, Lanes 13–15).

In RNA samples from the three gastric cancer cell lines examined, KATO-III, HSC-39, and MKN45 cells, a 156-base pair PCR product corresponding to the K-sam-II-type transcript and a 374-base pair product corresponding to the K-sam-I-type transcript were observed (Fig. 2A; Table 1). A 449-base pair product corresponding to the N-sam-type message was found in the two cell lines except for KATO-III. In RNA from the esophageal cancer cell line TE-1, all the three PCR products were detected, each corresponding to the K-sam-II-type, K-sam-I-type, and N-sam-type transcripts. In the five lung cancer cell lines, Lu-143, Lu-140, Lu-135, PC-10, and PC-3 cells, the K-sam-II-type transcript was found; in Lu-135 and PC-10 cells, the K-sam-I transcript was also detected. In the three lung cancer cell lines, PC-13, PC-1, and A549 cells, the K-sam-I-type message was not detected, whereas the K-sam-I-type message was observed. The N-sam-type transcript was detected in six of eight lung cancer cell lines; Lu-135 and PC-3 cells did not have the N-sam-type transcript.

In the analysis of the three sarcoma cell lines, A-172, G-361, and G-402 cells, neither K-sam-II-type nor K-sam-I-type transcript was identified. In RNAs from the two sarcoma cell lines, HT-1080 and RD cells, the K-sam-I-type PCR band was observed very weakly, but the band corresponding to the K-sam-II-type transcript was not detected (Fig. 2B). The N-sam-type transcript was present in all these sarcoma cell lines. In the immature teratoma cell line, NCC-IT, three bands corresponding to the K-sam-I-type, K-sam-II-type, and N-sam-type transcripts were observed. On the other hand, both the fetal brain and the adult brain expressed the K-sam-I-type and N-sam-type transcripts but not the K-sam-II-type transcript.

RNA Blot Analysis of the Third Ig-like Domain of the K-sam Transcript. To compare quantitatively the amount of the K-sam-II-type transcript with that of the K-sam-I-type transcript in carcinoma and sarcoma cell lines, RNA blot hybridization was performed using the specific probes, K7–3Ig and PK-3Ig (Fig. 1). We ascertained by the hybridization of these probes to the K-sam-II and K-sam-I cDNAs that these probes did not cross-hybridize to each other or to the N-sam sequence under high-stringency conditions (data not shown). In RNA blot analysis, the signal of the K-sam-II-type third Ig-like domain in KATO-III and HSC-39 cells was detected with higher intensity than that of TE-1 and MKN45 cells (Fig. 3), as expected from more than 50-fold amplification of the K-sam gene in the former two cell lines (6). The amount of the K-sam-II-type transcript was much more than that of the K-sam-I-type transcript in these four tumors. Two K-sam-II-type messages, 3.5 kilobases and 4.0 kilobases, were detected, each corresponding to the K-sam-II-type, K-sam-I-type, and N-sam-type transcripts were observed. On the other hand, both the fetal brain and the adult brain expressed the K-sam-I-type and N-sam-type transcripts but not the K-sam-II-type transcript.

Table 1 Tumor cell lines used for analysis for presence of K-sam and N-sam transcripts

<table>
<thead>
<tr>
<th>Cell</th>
<th>Origin</th>
<th>K-sam-II</th>
<th>K-sam-I</th>
<th>N-sam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KATO-III</td>
<td>Signet ring cell a.</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HSC-39</td>
<td>Signet ring cell a.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MKN45</td>
<td>Poorly differentiated adenoc.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TE-1</td>
<td>Well-differentiated squamous cell a.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lu-143</td>
<td>Small cell a.</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lu-140</td>
<td>Small cell a.</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lu-135</td>
<td>Small cell a.</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC-13</td>
<td>Large cell a.</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC-10</td>
<td>Moderately differentiated squamous cell a.</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>PC-3</td>
<td>Poorly differentiated adenoc.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC-1</td>
<td>Squamous cell a.</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A549</td>
<td>Bronchioalveolar adenoc.</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Sarcoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-172</td>
<td>Glioblastoma</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HT-1080</td>
<td>Fibrosarcoma</td>
<td>-</td>
<td>+</td>
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<td>RD</td>
<td>Rhabdomyosarcoma</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G-361</td>
<td>Malignant melanoma</td>
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<td>+</td>
</tr>
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<td>G-402</td>
<td>Leiomyoblastoma</td>
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<tr>
<td>NCC-IT</td>
<td>Immature teratoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Lanes

a The presence of K-sam and N-sam transcripts were analysed by RT-PCR; +, presence of the transcripts; -, presence of small amounts of the transcripts; –, absence of the transcripts.

b Ca., carcinoma, adenoc., adenocarcinoma.

K-sam-I, and N-sam cDNAs predict that the three products of RT-PCR are 156, 374, and 449 base pairs in size, respectively.

RNA Blot Analysis. Samples of 20 µg of total RNA were fractionated on a 1% agarose/formaldehyde gel and transferred onto the NitroPlus membrane (Micron Separations, Inc., Westboro, MA) as described (18). Hybridization was performed under a high-stringency condition at 42°C for 16-24 h in 50% formamide containing 0.65 M NaCl and was followed by washing in a buffer consisting of 0.1X standard saline citrate and 0.1% sodium dodecyl sulfate at 65°C for 1 h. Probes were K7–3Ig and PK-3Ig that were made by RT-PCR using the following primers: upstream primer, 5'–CACCTGGGG-GATAAATAGTIT'-3', and downstream primer, 5'–TGCTGTITGGCAG-GATAAATAGTIT'-3'. The probes hybridized specifically to the second half of the third Ig-like domain of the K-sam-II and the K-sam-I cDNAs, respectively.

Mitogenetic Assays. 2 × 10⁴ cells/well of TE-1 cells and A-172 cells were seeded in a 24-well plate (Nunc, Roskilde, Denmark) in RPMI 1640 with 7% FCS and Dulbecco's modified Eagle's medium with 10% FCS, respectively. After incubation for 24 h, the serum concentration in the medium was reduced to 0.01% for 48 h and 36 h, respectively. Increasing concentrations of a ligand protein, KGF or bFGF, were added to the culture medium at the concentration of 50 µg/ml of heparin (Sigma Chemical Co., St. Louis, MO). Transferrin was added to TE-1 cells in the concentration of 5 µg/ml. After incubation of TE-1 cells for 36 h or incubation of A-172 cells for 48 h, the cells were pulse-labeled with 0.5 µCi/ml [3H]thymidine (925 GBq/mmol; Amersham, Tokyo, Japan) for 6 h for TE-1 cells or for 4 h for A-172 cells. The cells were subsequently washed with phosphate-buffered saline, and 10% trichloroacetic acid-insoluble radioactivity was determined as described (19). Under the conditions used, the cells stayed alive during the [3H]thymidine incorporation assay as shown by trypan blue exclusion. We also examined the [3H]thymidine incorporation of esophageal cancer TE-10 cells in the same condition as in the TE-1 cells. MTT assay was performed to assess the growth stimulation of the cells by KGF. Briefly, 1 × 10⁴ TE-10 esophageal carcinoma cells were seeded in each well of a 96-well plate and cultured for 4 days in RPMI 1640 containing 0.7% FCS and various amounts ranging from 100 pg/ml to 100 ng/ml of KGF. MTT assay was performed using a kit available from Chemicon (Temecula, CA) according to the manufacturer's instructions.

K-sam EXPRESSION IN CANCER CELL LINES

Y. Hattori, unpublished data.
served in other cell lines, and they are considered to be generated by alternative splicing, the pattern of which could vary among different cells (15).

**Biological Response to Ligands.** To examine the responsiveness to KGF and bFGF of the cancer cells containing the K-sam or N-sam transcript, mitogenic assay was performed. TE-1 esophageal cancer cells, which contain predominantly larger amounts of the K-sam-II-type transcript over the K-sam-I-type message, showed increased thymidine uptake in response to KGF in a dose-dependent manner but did not show an apparent increase in thymidine uptake when the cells were cultured with bFGF. The experiment was repeated four times, and one typical result is presented (Fig. 4A). The KGF protein was clearly a potent mitogen for TE-1 cells with a half-maximal stimulation of the DNA synthesis at about 2 ng/ml. We also observed a similar increase in the [3H]thymidine incorporation for another esophageal cancer cell line, TE-10, and the cells showed growth stimulation assessed by MTT assay after culture with KGF (data not shown). On the other hand, the culture with bFGF enhanced thymidine uptake of A-172 glioblastoma cells expressing N-sam in a dose-dependent manner. The K-sam gene was not expressed in A-172 cells, and the cells did not show a response to KGF under the experimental conditions used (Fig. 4B).

**DISCUSSION**

Recently, it has been demonstrated that the K-sam-II-type transcript is present specifically in noncancerous epithelial cells (13, 15, 20). Here we showed that all cell lines that contained the K-sam-II-type transcript belonged to those derived from carcinomas. So far, there have been no sarcoma cells that express the K-sam-II-type transcript,
Fig. 2. RT-PCR analysis of K-sam and N-sam transcripts. K, band with the size of 156 base pairs using primers K7-7 and K7-11 showing the presence of the K-sam-II transcript; P, band size of 374 base pairs using primers PK-9 and PK-12 showing the presence of the K-sam-I transcript; N, band size of 449 base pairs using primers NS-10 and NS-13 showing the presence of N-sam. A, carcinoma cell lines. Lanes 1–3, KATO-III; HSC-39, and MKN45 gastric cancer cells, respectively; Lane 4, TE-1 esophageal cancer cells; Lanes 5–12, Lu-143, Lu-140, Lu-135, PC-13, PC-10, PC-3, and A549 lung cancer cells, respectively; Lane 13, K-sam-II cDNA; Lane 14, K-sam-I cDNA; Lane 15, N-sam cDNA. B, Lane 1, A-172 glioblastoma cells; Lane 2, HT-1080 fibrosarcoma cells; Lane 3, RD rhabdomyosarcoma cells; Lane 4, G-361 malignant melanoma cells; Lane 5, G-802 leiomyoblastoma cells; Lane 6, NCC-IT immature teratoma cells; Lane 7, human fetal brain; Lane 8, human adult brain.

even when searched for by the RT-PCR method using specific primers. Moreover, in some carcinoma cells containing the K-sam transcript, the amount of the K-sam-II-type transcript was much more abundant than that of the K-sam-I-type transcript, revealed by RNA blot analysis. The various amounts of the K-sam-I-type transcript were detected widely in both carcinoma and sarcoma cells. Recent studies revealed that the K-sam-II-type and K-sam-I-type messages are generated by an alternative splicing of the same K-sam gene (14, 15, 17). The molecular mechanism determining the carcinoma cell-specific splicing pattern remains to be elucidated.

Analysis of the DNA synthesis showed that TE-1 cells, in which the K-sam-II-type transcript was preferentially expressed, responded to KGF but not substantially to bFGF. KGF is produced from normal stromal fibroblast and, among the few known growth factors with mitogenic activity on the epithelial cells, KGF is specific to epithelial cells and has properties consistent with those of a major paracrine effector in epithelial cell proliferation (21). It is likely that stromal cells that produce and secrete the KGF protein interact with carcinoma cells containing the K-sam-II-type transcript to promote their proliferation. It should also be noted that many carcinoma cells produce bFGF (22–24), which presumably stimulates proliferation of stromal cells in paracrine manner.

Those tumor cells which respond to KGF may have a higher potential to proliferate and metastasize; an immunohistochemical study using the anti-K-sam antibody indicated that K-sam staining was positive only in the scattered population of cells in the primary region but diffusely positive in the metastasized sites. It is likely that K-sam-II-expressing carcinoma cells are selected positively during the process of the KGF-dependent cell growth. All the sarcoma cell lines examined here did not contain the K-sam-II-type transcript, whereas the K-sam-I-type and/or N-sam-type transcript was detected. The major ligand for both the K-sam-I-type and N-sam proteins has been reported to be bFGF. The A-172 cells that express only N-sam but not K-sam-I or K-sam-II showed the increased DNA synthesis in response to bFGF but not to KGF, suggesting that sarcoma cells showed bFGF-dependent growth but not KGF-dependent growth. The NCC-IT cells express the K-sam-I-type, K-sam-II-type, and N-sam messages simultaneously; this observation is congruous with the pleuripotent teratocarcinoma nature of this cell line (25), presumably with a mixed carcinoma and sarcoma phenotype.

It remains to be clarified why the TE-1 cells did not show significant mitogenic response to bFGF, although the cells expressed not only the K-sam-II-type but also the K-sam-I-type and N-sam transcripts. Because TE-1 cells express mRNA of bFGF but not KGF7, one possibility is that their high-affinity receptors for bFGF, K-sam-I and N-sam, are already saturated with endogenous bFGF, whereas the autocrine stimulation is not likely for the KGF signal transduction.

Fig. 3. RNA blot analysis of K-sam transcripts. Twenty μg of total RNA in each lane were hybridized with the 32P-labeled probes K7-Ig and PK-Ig specific to the third Ig-like domain of K-sam-II and K-sam-I transcripts, respectively. Lane 1, KATO-III; Lane 2, HSC-39; Lane 3, MKN45; Lane 4, TE-1.


7 S. Iida, unpublished data.
ACKNOWLEDGMENTS

The present studies indicated that carcinoma cells responded to KGF, while sarcoma cells responded to bFGF for their growth. It is possible that a specific blockade of ligand-receptor interaction by targeting to the second half of the third Ig-like domain may lead to development of a new approach to the growth suppression of cancer cells.

REFERENCES


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