FIGC, a novel FGF-induced ubiquitin-protein ligase in gastric cancers

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Abstract We have previously shown that fibroblast growth factor receptor 2 (FGFR2) plays an important role in gastric carcinogenesis. In this study, we have used a differential display approach to identify basic fibroblast growth factor (bFGF)-inducible genes in gastric cancer cells. Here, we report that one of these genes is predicted to encode a RING finger protein, designated FIGC. The *FIGC* gene was found to encode a polypeptide of 381 amino acids with a novel RING finger module at the NH_2-terminus and the COOH-terminal proline-rich region. Using an in vitro ubiquitination assay with recombinant protein, we demonstrate that FIGC has intrinsic E3 ubiquitin ligase activity and promotes ubiquitination. Our data indicate that *FIGC* upregulation in response to bFGF in gastric cancer might be implicated in carcinogenesis through dysregulation of growth modulator.

Keywords: Fibroblast growth factor; Ubiquitin; Gastric cancer; RING; Carcinogenesis; Fibroblast growth factor receptor 2

1. Introduction

Fibroblast growth factors (FGFs) are mitogens that regulate a multitude of cellular processes, including cellular proliferation, cellular differentiation, wound repair, angiogenesis, and carcinogenesis [1–6]. In addition, overexpression of FGF in various cell lines leads to phenotypic transformation [7–9]. FGFs stimulate cellular responses by binding to cell surface tyrosine kinase receptors [10]. Fibroblast growth factor receptors (FGFRs) encode a transmembrane tyrosine kinase receptor involved in signaling via interaction with the family of FGFs. Four structurally related receptors, designated isoforms of the FGFR-1, -2, -3, and -4, have been identified [11]. Basic FGF can bind with high affinity to the FGFR family members identified to date [12]. Although it is known that ligand binding stimulates FGF receptor tyrosine autophosphorylation and dimerization [12–14], the subsequent biochemical pathway responsible for FGF mitogenic signal transduction has not been elucidated.

We have previously shown that FGFR2 plays an important role in gastric carcinogenesis [6]. However, the mechanisms for the oncogenic activity of FGFs have not been identified. To further elucidate mechanisms of FGFs action, we have attempted to identify novel genes involved in the events of FGF-triggered signals using PCR-based differential display [15]. We are presently identifying and characterizing novel bFGF-inducible genes in gastric cancer cell line, SNU-16, in order to gain further insight into the role of bFGF in gastric cancer. In this paper, we describe the cloning and characterization of a cDNA representing a novel gene, *FIGC*, whose steady-state levels increased in response to bFGF.

Here, we describe a gene regulated by bFGF that encodes a predicted protein of 381 amino acids, which we named FIGC. We present evidence that FIGC contains a RING finger domain and has intrinsic E3 ubiquitin ligase activity. The RING motif consists of a series of eight conserved cysteines and histidines, which bind two zinc atoms and form a structure of "crossbraced" rings. Recent studies have shown that RING finger proteins play critical roles in mediating the transfer of ubiquitin to heterologous substrates as well as to the RING finger themselves [15–18]. Ubiquitin conjugation (ubiquitination) is recognized as a multifunctional signaling mechanism. Polyubiquitination is a signal for destruction of proteins by 26S proteasomes [19,20]. By serving as a signal for specific cellular protein degradation, ubiquitination plays a critical role in physiological regulation of many cellular processes. Hence, *FIGC* upregulation in response to bFGF in gastric cancer might be implicated in carcinogenesis through dysregulation of growth modulator.

2. Materials and methods

2.1. Cell culture

Human gastric cancer cell line, SNU-16 (Korean Cell Line Bank), was grown at 37 °C in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Life Technologies, Inc.) and a 1:100 dilution of a penicillin–streptomycin–fungizone solution (Life Technologies, Inc.). Subconfluent cells were incubated for 24 h in the above medium containing a reduced serum concentration (0.5%) to induce a relatively quiescent cell population. Cells were then either left untreated or treated for various times with 0.5% calf serum supplemented with 20 ng/ml recombinant human bFGF (Promega).

2.2. RNA isolation and cDNA synthesis

The TRI REAGENT kit (Life Technologies, Inc.) was used to isolate total RNA from cell lines. The first-strand synthesis of

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Abbreviations: bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; FGFR, FGF receptor; PBS, phosphate-buffered saline; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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single-strand cDNA from RNA for use as a PCR template was carried out with 1st Strand cDNA Synthesis Kit (Boehringer–Mannheim).

2.3. cDNA cloning and sequencing

GeneFishing kits (Seegene) were used for differential display following the manufacturer's instructions. The PCR or RACE products were cloned into TA cloning vector and then sequenced with T7 or SP6 promoter primers using DyeDeoxy terminators (Applied Biosystems) and an ABI 373 DNA sequencer (Applied Biosystems). The full-length cDNA sequence of *FIGC* was obtained using a 5′-RACE and 3′-RACE kit (Clontech) with human brain cDNA library (Clontech). Sequence identity was confirmed by BLAST searches on the combined GenBank/EMBL non-redundant (nr) and expressed sequence tag libraries (dbEST), accessed through the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov/). Protein homologies were traced using the Blitz algorithm run at the EMBL/European Bioinformatics Institute (http://www.ebi.ac.uk) and functional protein domains were traced using the BLOCKS WWW-server (http://www.blocks.fhcrc.org/).

2.4. Northern blot analysis

Northern blot analysis was carried out using a standard procedure. Cells were washed twice with ice-cold phosphate-buffered saline (PBS). Total RNA was isolated with the Trizol (Life Technologies, Inc.) one step total RNA isolation method according to the manufacturer's instructions. Briefly, 10 μg of total RNA was size-fractionated by gel electrophoresis in 1% agarose/6% formaldehyde gels and transferred onto the nitrocellulose membrane by a capillary blot method. Northern blots were prehybridized at 42 °C for 2 h with a solution containing 50% formamide, 5× SSC, 50 mM Tris–HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 100 μg/ml denatured salmon sperm DNA, and 0.2% bovine serum albumin before hybridizing with either ^{32}P-labeled *FIGC* cDNA probes (10^6 cpm/ml) in the same solution for overnight. The hybridized membranes were exposed to Fuji RX X-ray film at −70 °C.

Multiple tissue poly(a)+ RNA blots were purchased from Clontech (Palo Alto, CA) and hybridized according to the enclosed protocols. The ClonTech Multiple Tissue Northern blots contained 2 μg of oligo(dT)-purified mRNA from different specific normal human tissues.

2.5. In vitro ubiquitination assay

Ubiquitination assays were carried out by adding GST-FIGC (400 ng), rabbit E1 (50 ng, Calbiochem), UbcH5b (100 ng, Calbiochem), His-tagged ubiquitin (5 μM, Sigma) and 2 μl of bacterial lysate in ubiquitination buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, and 2 mM dithiothreitol (DTT). The reaction mixture (30 μl) was incubated at 30 °C for 2 h. The reactions were stopped with 2× SDS sample buffer, resolved on 10% SDS–PAGE and subjected to immunoblotting with an anti-His antibody (Santa Cruz Biotechnology).

2.6. GenBank accession numbers

p300 (*Homo sapiens*), Q09472; Utropin (Dystrophin-related protein 1, *H. sapiens*), CREB-binding protein-1 (*Caenorhabditis elegans*), P34545; Dystrophin-related protein 2 (*H. sapiens*), Q13474.

3. Results

3.1. Identification and sequence analysis of FIGC, a FGF-inducible gene

A partial cDNA fragment induced by bFGF was isolated by differential display using mRNAs from the bFGF-stimulated and unstimulated human gastric cancer cell line, SNU-16. Amplification products were displayed using agarose gel electrophoresis and ethidium bromide staining. However, DNA fragments, about 600 bp in size, were amplified to a greater degree when cDNA representing the RNA isolated from cells treated with bFGF was used as template. The 600-bp fragment was excised from the gel, reamplified, and subcloned. Nucleotide sequence analysis of the isolated cDNA and comparison with the GenBank data base indicated it to be a novel gene. Subsequent characterization of this gene in our laboratory revealed that it is induced by bFGF. Therefore, it has been designated FIGC (for bFGF-induced in gastric cancer).

For searching the full-length of the partial cDNAs, 5 RACE was performed by using both strands of the FIGC cDNA insert, which were sequenced as described in Section 2. The nucleotide sequence contained a long open reading frame encoding a protein of 381 amino acids with a predicted molecular mass of 42 kDa (Fig. 1). The cDNA clone contained a 375-nucleotide 3′-untranslated region with a consensus polyadenylation signal (the DDBJ/EMBL/GenBank™ Accession No. AB083199 and Swiss-Prot Accession No. Q9P0J7).

Northern blot analysis of RNA isolated from serum-starved or bFGF-treated SNU-16 cells was then performed to confirm the differential display results, indicating that FIGC was an bFGF-inducible gene. The FIGC transcript was upregulated following bFGF stimulation. FIGC RNA was increased by 4 h, was maximal by 8 h, and this high level was maintained throughout the experiment (Fig. 2).

3.2. FIGC contains a RING finger domain

A search of GenBank protein data bases showed that this protein has a novel RING finger motif at the NH_2-terminus

```
1    MSRHEGVSCDACLKGNFRGRRYKCLICYDYDLCASCYESGATTTRHTTDH   50
          •  •          •  •     •  •          •   •
51   PMQCILTRVDFDLYYGGEAFSVEQPQSFTCPYCGKMGYTETSLQEHVTSE  100
101  HAETSTEVICPICAALPGGDPNHVTDDFAAHLTLEHRAPRDLDESSGVRH  150
151  VRRMFHPGRGLGGPRARRSNMHFTSSSTGGLSSSQSSYSPSNREAMDPIA  200
201  ELLSQLSGVRRSAGGQLNSSGPSASQLQQLQMQLQLERQHARAARQQLET  250
251  ARNATRRTNTSSVTTTITQSTATTNIANTESSQQTLQNSQFLLTRLNDPK  300
301  MSETERQSMESERADRSLFVQELLLSTLVREESSSSDEDDRGEMADFGAM  350
351  GCVDIMPLDVALENLNLKESNKGNEPPPPPL*
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Fig. 1. Predicted amino acid sequence for human *FIGC*. The deduced amino acid sequence is shown and numbered starting at the presumable initiation methionine. Six conserved amino acids in the RING finger domain (●) are shown below amino acid sequence and proline-rich regions are *underlined*.

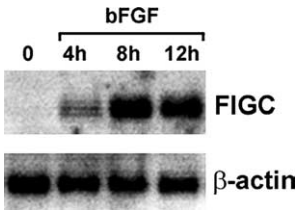

Fig. 2. Effect of bFGF on *FIGC* mRNA levels. Serum-starved cells were either left untreated or treated with bFGF (10 ng/ml) for the times indicated. Total RNA (10 μg) was subjected to Northern blot analysis as described under Section 2. β-Actin was used as an internal standard for quantification of total RNA in each lane of the gel.

(Fig. 3A). RING finger proteins were previously classified into the C3HC4 RING fingers and C3H2C3 RING fingers [21]. In this study, FIGC, p300, Utrophin, Creb-binding protein-1 (CBP-1), and Dystrophin-related protein2 (Drp2) were classified into the novel subclass of RING fingers, which contains distinguished consensus sequence **C**X2**C**(7-11)**C**X2**C**X_A5-**C**X2**C**X(5-9)**H**X(1-3)**H** (X_A: acidic residues) from C3HC4 RING fingers and C3H2C3 RING fingers. All eukaryotes encode C6H2-type RING proteins and such proteins were represented in some species by several distinct isolates, indicating that the C6H2-type RING finger unit defines an evolutionarily highly conserved module (Fig. 3).

3.3. FIGC has ubiquitin-protein ligase activity

The RING finger motif is essential for the E3 activity. It has been shown that the RING finger motif is required for the ubiquitin ligase activity for almost all the RING finger-containing E3 enzymes. E3 accelerates the ubiquitination of its substrate and sometimes E3 is itself ubiquitinated. Therefore, the in vitro demonstration of substrate-independent auto-ubiquitination is a good indication of the E3 activity. We decided to examine whether FIGC has intrinsic ubiquitin-protein ligase activity. To determine if FIGC has intrinsic ubiquitin-protein ligase activity, we used an in vitro ubiquitination assay. Affinity purified GST-FIGC was incubated at 30 °C with ATP, His-tagged ubiquitin, and the bacterial lysates containing recombinant E1 and E2, and subjected to immunoblotting with the anti-His antibody. As shown in Fig. 4, FIGC-mediated ubiquitination was readily detected and was dependent on the

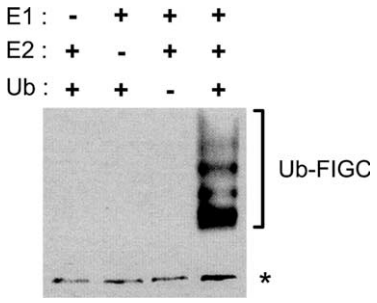

Fig. 4. Ubiquitination of FIGC. GST-*FIGC* was evaluated for E3 activity in the presence of His-tagged Ub as indicated. Following the ubiquitination reaction, the samples were subjected to SDS–PAGE and immunoblotting with His antibody to reveal ubiquitinated products. An *asterisk* marks the non-specific bands observed in all lanes.

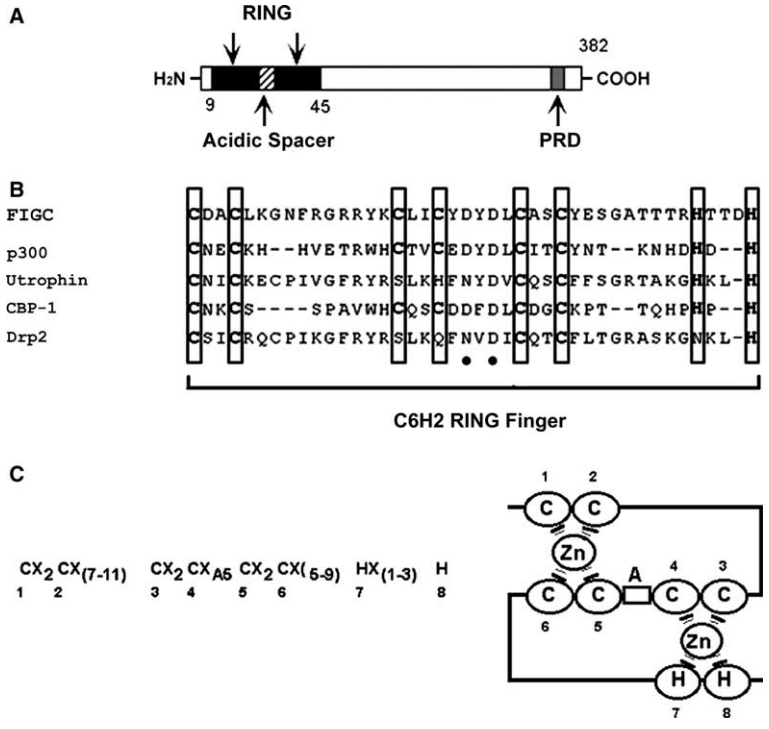

Fig. 3. Schematic presentation of *FIGC* cDNA. (A) The coding regions are depicted as an open box. RING finger domain (closed box), acid spacer element (hatched box), and proline-rich region (gray box) in ORF are shown. (B) Amino acid alignments of RING finger modules. Alignments of the amino acid sequences of the C6H2 regions of *FIGC* and proteins containing *FIGC*-type zinc fingers from a range of different species are shown. Putative metal-ligating residues (based on their conservation across all species) are highlighted in bold, and the consensus sequence is boxed. C6H2 RING finger is separated by an acidic spacer element (●). (C) C6H2-type RING finger consensus sequence and schematic presentation of *RING finger*.

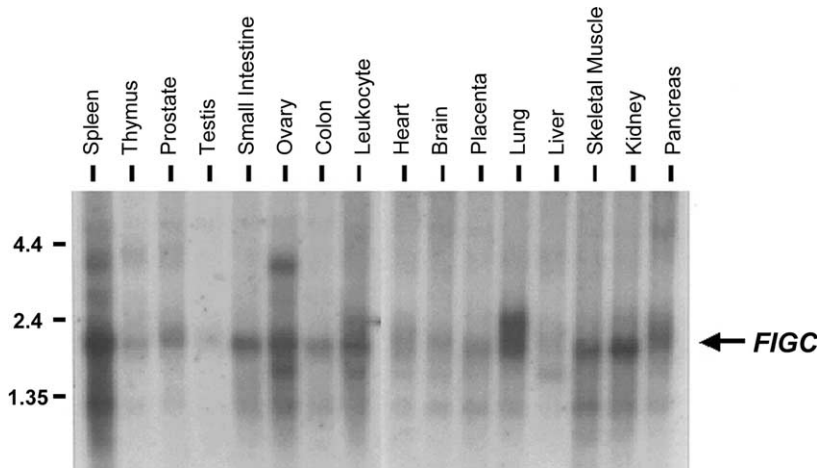

Fig. 5. Northern blot analysis of *FIGC*. Polyadenylated RNA from different tissues on Northern blots was hybridized with a probe of full-length cDNA. The *arrow* indicates the hybridizing band of approximately 2 and 1 kb.

presence of E1 and E2. The results suggested that FIGC in fact had the ubiquitin-protein ligase activity.

3.4. Messenger RNA expression of FIGC in human organs

We next used Northern blot analysis to examine the tissue distribution of *FIGC* mRNA. The *FIGC* gene was expressed in human spleen, small intestine, ovary, peripheral blood, lung, skeletal muscle, kidney, and pancreas, but was expressed at a low level in the thymus, prostate, testis, colon, heart, brain, placenta, and liver (Fig. 5).

To date, very few FGF response genes have been identified [22]. Accordingly, we have utilized the differential display methodology to isolate cDNAs representing such genes [23–25]. In this paper, we describe the cloning and characterization of a cDNA representing a novel gene, *FIGC*, whose steady-state levels increased in response to bFGF. We show that the FIGC protein has a novel C6H2-type RING finger domain at the NH_2-terminal region. To date, all characterized examples of RING finger domains have been shown to possess E3 ligase activity in vitro [21,26,27]. For some RING finger E3 ligases, it was found that the RING finger structure binds the E2. However, the exact mechanism by which the RING finger containing E3 ligase catalyzes the transfer of ubiquitin to the target proteins is yet unknown.

Although we have shown here that FIGC is polyubiquitinated, probably signifying that it functions as an E3 ligase, we cannot exclude other potential functions. Indeed, accumulating evidence has shown that RING fingers have other cellular functions. Zinc-coordinated RING fingers have been shown to mediate dimerization of proteins such as BARD1 and BRCA1, Mdm2 and MdmX, and constitutive photomorphogenic protein 1and CIP8 [28–30]. Recently, a RING finger-like domain, the FYVE domain, has been determined to bind specifically phosphatidylinositol 3-phosphate [31]. The FYVE domain contains a highly conserved motif that provides specificity for binding to phosphatidylinositol 3-phosphate. Moreover, in the case of Mdm2 the RING finger not only mediates ubiquitination for p53 but also binds to RNA [32].

FGFR represents an important example of a single gene that causes different human developmental and tumoral diseases. We have previously identified two identical mutations in *FGFR2* that cause craniosynostosis syndromes, Crouzon, Apert, and Pfeiffer in gastric cancers [6]. The finding of identical mutations in skeletal dysplasias and gastric cancers is the first evidence for the involvement of the somatic mutations of *FGFR2* in gastric cancer, and suggests that a similar mechanism of ligand-independent constitutive activation of the *FGFR2* product is responsible for both diseases. Thus, the identification of substrates of *FIGC* in response to bFGF signal should greatly advance our understanding of the tumorigenic activity of FGFs. Further studies are in progress to identify and characterize proteins interacting with FIGC and to knock out the *FIGC* gene.

Thus, upregulation of *FIGC* might affect the turn-over rates of its substrates of ubiquitination and might lead to dysregulation of negative regulators of tumor development in gastric cancer. Although the substrates of FIGC remain to be elucidated, upregulation of *FIGC* in response to bFGF in gastric cancer might be implicated in carcinogenesis through dysregulation of growth modulator. Identification of its substrates may provide a potential new target for assessing the tumorigenic activity of FIGC or FGFs in tumor therapy.

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