Cloning of a G-protein-coupled receptor that shows an activity to transform NIH3T3 cells and is expressed in gastric cancer cells

Shun-ichiro Okumura,[1, 2] Hiroko Baba,[1, 3] Tatsuro Kumada,[1] Koji Nanmoku,[1] Hirofumi Nakajima,[1] Yasushi Nakane,[2] Koshiro Hioki[2] and Kazuhiro Ikenaka[1, 4]

[1]Laboratory of Molecular Neurobiology, National Institute for Physiological Sciences, Okazaki National Research Institutes, Myodaiji-cho, Okazaki, Aichi 444-8585; and [2] Department of Second Surgery, Kansai Medical University, Moriguchi, Osaka 570-8506

(Received October 23, 2003/Revised December 2, 2003/2nd Revised December 22, 2003/Accepted December 22, 2003)

The present study was directed towards the identification of novel factors involved in the transformation process leading to the formation of gastric cancer. A cDNA library from human gastric cancer cells was constructed using a retroviral vector. Functional cloning was performed by screening for transformation activity in transduced NIH3T3 cells. Six cDNA clones were isolated, including one encoding the elongation factor 1α subunit, which was already known to play a role in tumorigenesis. One cDNA (clone 56.2), which was repeatedly isolated during the course of screening, encoded a protein identical to a G-protein-coupled receptor protein, GPR35. In addition, another cDNA clone (72.3) was found to be an alternatively spliced product of the *GPR35* gene, whereby 31 amino acids were added to the N-terminus of GPR35. Hence, the proteins encoded by clones 56.2 and 72.3 were designated GPR35a and GPR35b, respectively. RT-PCR experiments revealed that *GPR35* gene expression is low or absent in surrounding non-cancerous regions, while both mRNAs were present in all of the gastric cancers examined. The level of 72.3-encoded mRNA was consistently significantly higher than that of 56.2 encoded mRNA. An expression pattern similar to that observed in gastric cancers was detected in normal intestinal mucosa. Based on the apparent transformation activities of the two GPR35 clones in NIH3T3 cells, and the marked up-regulation of their expression levels in cancer tissues, it is speculated that these two novel isoforms of GPR35 are involved in the course of gastric cancer formation. (Cancer Science 2004; 95: 131–135)

Gastric cancer is one of the most frequently occurring cancers in Japan, with mortality rates as high as 40 per 100,000 individuals per year. Histologically, gastric cancers can roughly be grouped into well differentiated type and poorly differentiated type adenocarcinomas. Although both types arise from the same gastric mucosa, they differ in various aspects, including the progression rate, invasive character and sensitivity to chemotherapeutic agents.[1, 2] It is therefore crucial to understand the molecular mechanism(s) underlying the transformation of normal mucosa to each of the different types of adenocarcinoma to develop the most effective treatments. During the precancerous stage, well differentiated type cancer is often associated with intestinal metaplasia while the poorly differentiated type is not.[3] Hence, the two types are distinguishable at early stages of cancer formation. Various abnormalities have been reported that are common to both types (genetic instability, over-expression of TGFα, inactivation of p53, etc.)[4, 5] or that occur predominantly in one type (inactivation of *adenomatous polyposis coli* gene, amplification of *K-sam* gene, etc.).[6, 7] Nevertheless, elucidation of the entire pathway of normal mucosal transformation to gastric cancer is far from complete.

The present study was directed towards the identification of novel factors involved in the transformation process leading to the formation of gastric cancer. A cDNA library was constructed from gastric cancer cells using a retroviral vector. Because the retroviral vector integrates into the host chromosome in a single copy, this enables screening for the desired cDNA clone without repeating the first screening steps.[8] This retroviral cDNA library was used to transduce NIH3T3 fibroblasts. Many colonies of cells that had lost contact inhibition were purified and expanded. In most cases, recovery of the cDNA inserts from these colonies resulted in the isolation of single cDNA clones, as expected. One cDNA clone was repeatedly recovered from many independently transformed colonies, implying that expression of this gene is inherently related to the transformation process. Sequence analysis revealed that this cDNA encodes a member of the G-protein-coupled receptor (GPCR) family previously reported as GPR35, whose function or ligand was unknown.[9] The *GPR35* gene potentially produces another related protein through alternative transcription initiation site usage. This gene was expressed in all the gastric cancers examined and expression was always more abundant in cancer tissues than in surrounding non-cancerous mucosa. These results indicate that *GPR35* gene expression is coupled with the formation of gastric cancer.

Materials and Methods

cDNA library construction. Total RNA from human gastric cancer tissue was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method.[10] Poly(A)$^+$ mRNA was isolated using the Messenger RNA Isolation Kit (Stratagene). cDNA was synthesized from Poly(A)$^+$ mRNA using the SuperScript Plasmid System (Gibco-BRL). The retroviral vector, pHBR299$^+$, was constructed from pTYR299$^+$ 11) by modifying the cloning sites to allow directional insertion of the library cDNAs. This vector contains an extended packaging signal region between the long terminal repeats (LTRs) to obtain high infectious titers and was digested with *Not*I and *Sal*I to allow directional cloning. The synthesized cDNA library was inserted into this site with a Ligation high kit (Toyobo). The study protocol conformed to the Helsinki Declaration of 1975.

Amplification of the library. Ten percent glycerol aliquots of *Escherichia coli* DH10B electrocompetent cells (Gibco-BRL) were transformed with the cDNA library in pHBR299$^+$ using a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's recommendations. The electroporated cells were dispensed into SOC medium, and grown for 1 h at 37°C. The transformed cells were plated out on LB-ampicillin plates (100 μg/ml), and incubated overnight at 37°C. Then colonies were

[3]Present address: Department of Molecular Neurobiology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392.
[4]To whom correspondence should be addressed. E-mail ikenaka@nips.ac.jp

scraped with the aid of added LB medium from the plates, and plasmid DNA was prepared using the Plasmid Giga Kit (Qiagen).

Transfection of packaging cell line. An ecotropic retrovirus packaging cell line ψMP34, established from ψ2 by stably introducing the polyomavirus early gene, yields titers 10–100 times higher than those of the parent cell lines.[11] ψMP34 was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS). The cells were seeded (2×10^5 cells) onto 6-cm dishes 1 day before transfection. Cells were transfected with plasmid DNA using LipofectAMINE Reagent (Gibco-BRL) according to the manufacturer's protocol. Plasmid DNA (4 μg) was mixed with 20 μl of LipofectAMINE Reagent in 400 μl of serum-free DMEM. After 45 min at room temperature, 1.6 ml of serum-free DMEM was added, and the mixture was layered onto the cells, which had been rinsed with serum-free DMEM. Six hours later, the medium was replaced with 4 ml of fresh DMEM containing 10% FCS (DMEM/FCS).

Infection of NIH3T3 fibroblast cells and determination of viral titer. The cells were incubated in 5% CO_2 in air at 37°C for 48 h, then the virus-containing supernatant was harvested from the culture dishes, filtered through a 0.45 μm filter, and immediately used for infection of NIH3T3 fibroblast cells. One milliliter aliquots of the virus-containing supernatant, mixed with 4 ml of fresh DMEM/FCS and Polybrene (a final concentration of 8 μg/ml), were used to infect NIH3T3 cells plated the previous day at 5×10^5 cells per 10-cm dish. After 4 h incubation, 2.5 ml of medium was removed, and 7.5 ml of fresh DMEM/FCS was added to decrease the concentration of Polybrene (a final concentration of 2 μg/ml). To estimate the virus titer, NIH3T3 cells were infected with a recombinant retrovirus containing the *lacZ* gene. The infected cells were detected by X-Gal staining,[12] and the titer was estimated according to the following formula: Titer of retrovirus (cfu/ml)=number of X-Gal-positive cells/virus volume (ml)×4 (replication factor of NIH3T3).[13]

Focus isolation. The culture was maintained at 37°C in 5% CO_2 with twice- or three times-weekly feeding of DMEM/FCS+ for 3 weeks to confirm the emergence of transformed foci. Foci of transformed cells were picked up and trypsinized to seed onto new dishes. Two weeks after re-seeding, emerged foci were re-picked and seeded onto 22-mm dish to expand them for genomic DNA preparation.

Genomic DNA preparation and PCR recovery of cDNA inserts. Expanded cells were lysed with SDS, and genomic DNA was prepared by proteinase K digestion, phenol extraction and isopropanol precipitation. cDNA segments were amplified by using upstream and downstream retroviral vector primers (5′ primer, TTGAACCTCCTCGTTCGACC; 3′ primer, GAGCCTGGACCACTGATATC). The PCR reaction was run for 30 cycles (20 s at 98°C, and 10 min at 68°C) using the LA PCR Kit (TaKaRa). The resulting PCR fragment was cloned into the pCR 2.1 vector (Invitrogen) and sequenced using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) carried out on an Applied Biosystems model 377 sequencer. cDNA in pCR 2.1 was subcloned into the pHBR299$^+$ vector to confirm its transforming activity. NIH3T3 cells were infected with the viral supernatant, and the culture was maintained in DMEM/FCS to confirm the emergence of transformed foci.

RT-PCR. Total RNA was isolated from clinical specimens using TRIzol reagent (Gibco-BRL), and 1 μg was taken for cDNA synthesis using Superscript II RNase H$^-$ Reverse Transcriptase (Gibco-BRL) and a random nanomer primer; pd$(N)_9$ (TaKaRa). For semi-quantitative studies, the amount of cDNA used as the PCR template was always normalized against β-actin expression levels. The appropriate cycle number was determined for each primer set and the conditions were maintained throughout this analysis.

Results

Isolation of cDNAs with transforming activity from a human gastric cancer cDNA library. To isolate cDNAs that functionally regulate tumorigenesis in the stomach, expression cloning was performed using a retroviral cDNA library constructed from human gastric cancer tissue. A total of 1.4×10^6 independent clones was screened and 45 cDNAs were isolated based on their abilities to transform NIH3T3 cells. These isolated clones were partially sequenced using primers that hybridized to sequences within the viral vector, and the sequences were compared to one another. Twenty-eight independent cDNA clones were obtained. Of these, 15 clones that contained unknown cDNA inserts larger than 1 kb or full coding regions of known genes were selected for further examination.

The inserts were subcloned again into the viral vector pHBR299$^+$ and were transfected independently into a viral producer cell line to obtain retroviral vectors. For the second screening, each cDNA clone was transduced to NIH3T3 cells to test transformation activity.

Table 1 summarizes the properties of 6 clones that repeatedly exhibited apparent transforming activities. Based on sequence analysis, four clones (67.3, 70.5, 75.1 and 86.3) were found to be identical to known genes encoding phosphatidylglycerophosphate syntase (PGS1, lacking a portion of the N-terminal sequence), HLA-Cb-2, the elongation factor 1α subunit and gut Kruppel-like factor (GKLF), respectively. Since the elongation factor[14] and GKLF[15] are known to be involved in tumorigenesis, the screening method utilized was proven to be appropriate for the current study. In contrast, comparison of the partial sequences of two cDNAs (56.2 and 72.3) with the GenBank database revealed no significant homologies with any known genes. Interestingly, sequence comparison between clones 56.2 and 72.3 revealed identical sequences in the respective 3′ ends, while the 5′ ends of these clones displayed no homology at all. These results suggest that the two clones are highly related, and are most likely alternatively spliced products from the same gene. Furthermore, five additional clones with the same sequence as clone 56.2 were isolated independently during the first screening, suggesting that this mRNA was either highly abundant in the gastric cancer cDNA library or the transforming activity of this cDNA was very high. Based on these interesting properties, clone 56.2 and its related clone 72.3 were chosen for further characterization.

Two overlapping cDNA clones encode new isoforms of GPCRs. Se-

Table 1. Summary of cDNA clones with transformating activity

Clone	Size (kb)	Results of BLAST search	Same clones
56.2	2.3	GPR35	11.1/12.3/81.1/83.2/83.3
67.3	2.2	Phosphatidylglycerophosphate synthase	13.1/75.2
70.5	1.1	HLA-Cb-2	70.1
72.3	2.5	GPR35 related gene	—
75.1	1.7	Elongation factor 1 α subunit	—
86.3	2.3	Gut Kruppel-like factor	—

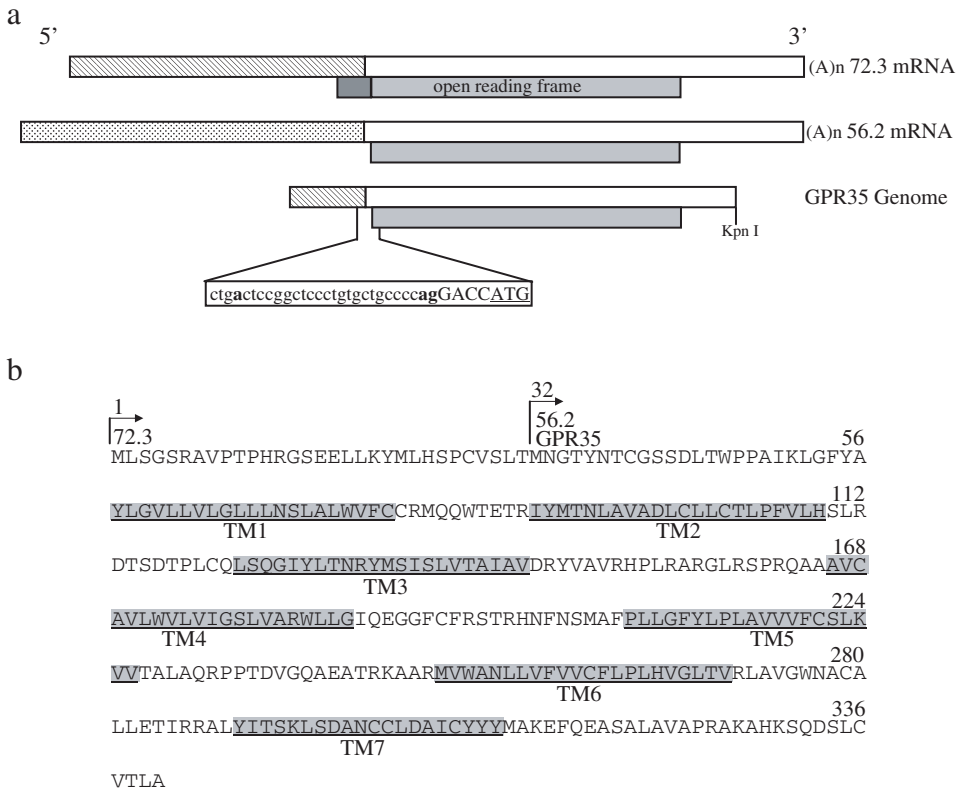

Fig. 1. Schematic diagrams of the mRNA structures corresponding to clones 56.2 and 72.3. a) Comparison of the mRNA structures of clones 56.2 and 72.3, as well as the published genomic structure of GPR35. Because 56.2 and 72.3 have common 3′ regions (white boxes) with completely unrelated 5′ regions (dotted or oblique lines), and consensus splicing signals were found at the junctional region in the corresponding genomic sequence of GPR35, these two clones were most likely produced through alternative exon usage from a single *GPR35* gene. The genomic sequence of the junctional region is indicated below. The sequences represented by small letters correspond to the intron, while those represented by capital letters indicate the exon. The underlined ATG is the translational initiation signal for 56.2 mRNA. The bold face "a" indicates the branch point for lariat formation, and the bold face "ag" represents the splice acceptor signal. b) The deduced amino acid sequences of GPR35-related proteins. Both proteins contain the common 7 transmembrane (TM) protein structure, which is characteristic of G-protein coupled receptors (GPCR). The arrows above the sequence indicate the translation initiation sites of the two putative protein products produced from 72.3 and 56.2. The clone 56.2 encodes the same protein as the previously reported *GPR35* gene, while an additional 31-amino-acid sequence is present in the N-terminal region in the protein encoded by 72.3. The underlined shaded sequences indicate the 7 TM portions. The numbers on the right indicate the amino acid numbers based on the translation pattern of 72.3.

quence analysis revealed that clones 56.2 and 72.3 contained insert lengths of 2307 and 2172 bp, respectively. A schematic diagram of the mRNA structures for 56.2 and 72.3 (Fig. 1a) shows that the 3′-terminal 1300 bp regions of these two clones are identical. A GenBank search revealed that the nucleotide sequence shared by these two clones is identical to the published gene encoding a GPCR, GPR35. The *GPR35* gene was originally isolated from a human genomic library during an effort to identify the open reading frames (ORFs) of novel genes encoding GPCRs.[9] Translation of GPR35 protein was reported to be initiated from the ATG indicated in Fig.1a. Interestingly, a common DNA sequence spanning from 4 nt upstream of this initiation codon to the reported 3′ terminus of the *GPR35* gene is found among clones 56.2 and 72.3, and the *GPR35* gene. In contrast, the upstream regions were completely unrelated, and a BLAST search failed to reveal homology with any known gene in the database. To determine whether the distinct 5′ upstream regions of the two clones were a result of splicing, the junction point in the GPR35 genomic sequence was searched for potential splicing signals. The consensus sequences for intron-exon boundaries, including, a branch point for lariat formation, a polypyrimidine stretch and the splice acceptor signal "AG" at the 3′ end, were identified at the junction region in the *GPR35* gene. Therefore, in clone 56.2, translation most likely initiates from the ATG codon published for GPR35, while an additional in-frame ATG occurs in the upstream region of clone 72.3, suggesting that the protein translated from clone 72.3 contains an additional 31-amino-acid sequence at the N-terminus of the protein encoded by clone 56.2 (Fig. 1b). Thus, the *GPR35* gene produces at least two mRNAs, most probably through alternative splicing, and these two novel isoforms are implicated in the transformation of the fibroblast cells.

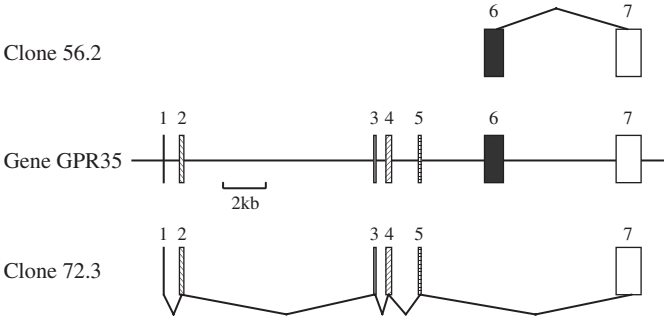

Fig. 2. Structure of the *GPR35* gene and its exon usage. A homology search of the cDNA sequences of 56.2 and 72.3 against the human genomic sequence was performed. This gene was located at human chromosome 2q 37.3 (GenBank Accession number AF158748) and the exon-intron boundaries were determined by comparing the sequences of cDNA and the genome. Exons are indicated as open boxes and introns as lines. The original *GPR35* gene is located on exon 7.

Structure of the *GPR35* gene. To show more directly that the two related cDNAs are alternatively spliced gene products, we determined the gene structure of *GPR35*. Homology search of the cDNA sequences against the human genomic sequence was performed. This gene was located at human chromosome 2q 37.3 (GenBank Accession number AF158748) and the exon-intron boundaries have been determined as shown in Fig. 2. It

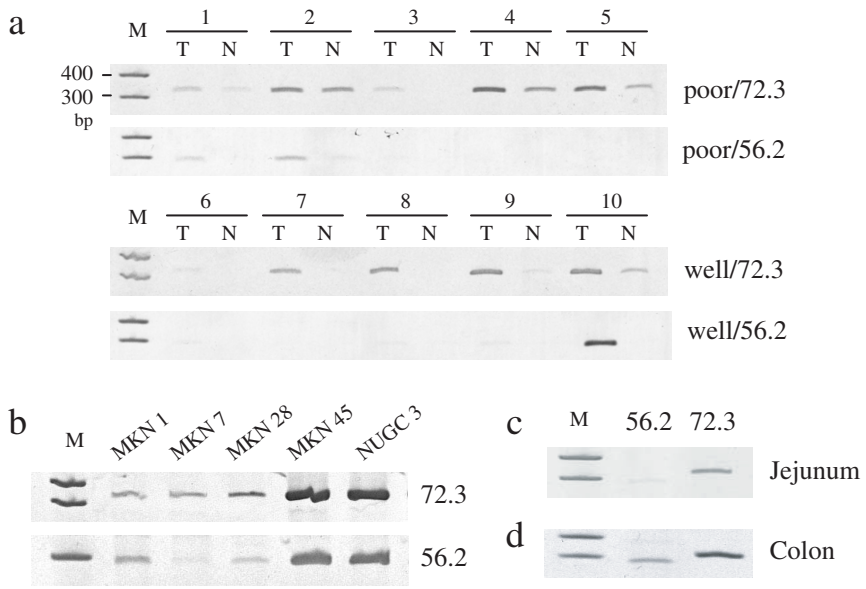

Fig. 3. The expression of GPR35-related mRNAs in human tissues. a) The 72.3-related mRNA was detected in all of the gastric cancers examined irrespective of well or poorly differentiated type. The expression of 56.2 mRNA was lower than that of 72.3 in all samples and this mRNA was not detected in the normal surrounding areas, with the exception of sample #10. The 72.3 mRNA was expressed in the tissues surrounding poorly differentiated cancer, although the expression levels were always significantly less than those in cancers. b) Expression of *GPR35* gene was studied in several gastric cancer cell lines (MKN1, MKN7, MKN23, MKN45, NUGC3). Both mRNAs were detected in all the cell lines examined. c) The expression of 72.3-related mRNA in the normal human small intestine (jejunum) was nearly the same as that observed in gastric cancer cells. The level of 56.2-related mRNA was far less than that of 72.3-related mRNA. d) Expression of *GPR35* gene was studied in human colon. Both mRNAs were detected in colon, but the level of 56.2-related mRNA was much abundant than in intestine.

consisted of seven exons and the common sequence was encoded by the last exon, which had been identified as the *GPR35* gene previously.[9] The mRNA encoding 56.2 utilized exons 6 and 7, while that of 76.2 contained exons 1 to 5, and 7. In view of this gene organization, it is likely that the two mRNA species were produced by alternative transcription initiation site usage.

Two novel isoforms of GPR35 are predominantly expressed in tumor tissues. The expression levels of mRNAs corresponding to clones 56.2 or 72.3 were examined in the gastric mucosa, which was surgically removed from patients together with the gastric cancer. Each message was detected by the RT-PCR method using specific primer sets, and the expression levels were compared in well or poorly differentiated tumor tissues, as well as in the surrounding non-cancerous regions from the same sample. PCR products specific for 72.3 (320 bp) and 56.2 (283 bp) were detected in tumors, indicating that these two cDNAs were not cloning artifacts but were expressed as actual transcripts in the human stomach (Fig. 3a). For semi-quantitative studies, the amount of cDNA used as the PCR template was always normalized using the human actin primer set, and appropriate cycle numbers were determined for each primer set (33 cycles for 56.2 and 30 cycles for 72.3). These conditions were maintained throughout this analysis. The 72.3-related mRNA was detected in all of the gastric cancers examined irrespective of whether they were well or poorly differentiated (Fig. 3a). Non-cancerous regions were also obtained from the edge of the surgically removed regions, where no apparent invasion by the tumor cells were observed. Interestingly, this mRNA was also expressed in the tissues surrounding poorly differentiated cancer, although the expression levels in the surrounding tissues were always significantly less than those detected in cancers. In well differentiated tumors, however, 72.3-related mRNA was not detected in the surrounding tissues in 3 of 5 patient samples (Fig. 3a, #6, 7, 8). RT-PCR using specific primer sets for 56.2 demonstrated that the expression levels were lower than those of 72.3 in all samples (note the difference in the cycle number between 56.2 and 72.3) and this mRNA was not detected in most of the normal surrounding areas. Taken together, these results suggest that both types of GPR35-related proteins are prominently expressed in tumor tissues, and may function in the formation of gastric cancers.

To confirm the expression in cancer cells, GPR35 expression in several gastric cancer cell lines were studied. Fig. 3b shows that it is expressed in all the cell lines examined.

Expression of the *GPR35* gene in the intestine. Since intestinal metaplasia frequently accompanies gastric cancer and it has been reported that the *GPR35* gene is expressed in the intestine,[9] RT-PCR was performed on total RNA isolated from human intestine and colon employing the same conditions used for the analysis of *GPR35* gene expression in gastric cancers. The amount of 72.3-related mRNA in the small intestine was nearly equal to or even higher than that in gastric cancers. The level of 56.2-related RNA was far less than that of 72.3-related RNA; nevertheless, it was still detectable (Fig. 3c). Hence, the levels and patterns of *GPR35* gene expression in the normal small intestine are quite similar to those observed in gastric cancers. The level of *GPR35* gene expression was also similar in colon (Fig. 3d), though 56.2-related RNA was much more abundant in colon.

Discussion

GPR35 was originally identified as a putative protein encoded by an ORF discovered during a genomic library screen; there was no information on the structure of GPR35 mRNA.[9] The present study demonstrates the existence of at least two types of GPR35 mRNA, with 5′ regions that are distinct from the genomic sequence and fused to common 3′ regions with sequences corresponding to the genomic 3′ sequences. These additional sequences corresponded to exons 1 to 5 or to exon 6 (Fig. 2). The previously identified ORF resides on one exon (exon 7). In one mRNA (corresponding to clone 72.3) the ORF extended further upstream from the original translation initiation site, revealing a new ATG codon which would yield a protein with 31 additional amino acids at the N-terminus of the GPR35 protein previously reported.[9] The protein encoded by the other mRNA (corresponding to clone 56.2) is identical to the previously reported GPR35 protein. Hence, the original GPR35 protein encoded by the mRNA corresponding to clone 56.2 has been designated “GPR35a,” and the newly found putative protein with 31 extra amino acids is designated “GPR35b.”

Both mRNAs possess long 5′-untranslated regions; 1008 nucleotides for clone 56.2 and 774 nucleotides for clone 72.3. The significance of these long untranslated sequences remains to be determined. It is possible that these sequences control the efficiency of translation initiation, but we do not know the protein levels of the two forms at present.

When expressed in NIH3T3 fibroblasts, both clones transformed the cells, causing them to lose contact inhibition and form cell aggregates. Thus, it may be surmised that mitogenesis is stimulated by the presence of GPR35a and b. It has been demonstrated that many GPCRs transactivate epidermal growth factor receptor, and stimulate mitogenesis.[16–18] Importantly this phenomenon has also been demonstrated to occur in colon cancer and gastrointestinal hypertrophy.[19] These data, together with the observation that the *GPR35* gene is expressed more abundantly in gastric cancer cells than in the surrounding mucosa, suggest that GPR35a and b may function in cell growth control. Nevertheless, the possibility that aberrant expression of the *GPR35* gene in fibroblasts leads to the unusual coupling of GPR35 to cell growth cannot be ruled out, and hence the true function of GPR35a and b in gastric cancers, as well as in the mucosa of intestine and colon needs to be clarified.

The appearance of transformed NIH3T3 cells was observed 10 days after retroviral transduction of 56.2 cDNA whereas no transformed phenotypes were observed at this stage for cells transduced with 72.3 cDNA (data not shown), indicating that the transforming activity of 56.2 is stronger than that of 72.3. These results suggest that the truncation of the N-terminus in GPR35a creates a constitutively active receptor, which is similar to what has been observed for the thrombin receptor.[20] The N-terminal region of the thrombin receptor is proteolytically cleaved to create a constitutively active form while the truncated form of GPR35b is produced by alternative transcription initiation site usage.

It has been reported that the *GPR35* gene is expressed in the rat intestine, but not in heart, spleen, liver, lung, ovary, kidney or whole brain. Expression in human brain, lung and adrenal was also not detected.[9] The present study reveals that GPR35 is occasionally expressed in human gastric mucosa surrounding cancer tissues and abundantly expressed in intestinal mucosa, as well as in stomach cancer. Thus, expression of this gene is quite specific to the mucosa of intestine and colon within normal tissues. While it would be of interest to determine whether this gene is expressed in normal human gastric mucosa, the observation that GPR35 expression was undetectable in some gastric mucosa surrounding the cancer tissue suggests that it is not expressed under normal conditions. It is likely that the expression of this intestine-specific gene is one of the phenomena linked to the precancerous changes leading to the formation of stomach cancer.

It is intriguing to speculate that the signaling pathway coupled to this GPCR in the intestinal mucosa is distinct from that in the gastric mucosa and the expression of GPR35 is tumorigenic only in the gastric mucosa. It is also possible that the ligand levels of this receptor in the intestine or colon are different from those in the stomach, which may cause unregulated cell growth only in the gastric mucosa.

Gastric cancers are often associated with intestinal metaplasia.[21, 22] However, the link between the two is still unclear. The results of the present study demonstrated that the intestine-expressed *GPR35* gene is missexpressed in 6 out of 10 non-cancerous tissues surrounding gastric cancers (Fig. 3a). Thus, GPR35 expression in gastric tissue may play an important role in gastric cancer formation. It would be of interest to examine the relationships among expression of the *GPR35* gene, intestinal metaplasia and gastric cancer.

This work has been supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

1. Maehara Y, Kusumoto T, Emi Y, Anai H, Sakaguchi Y, Kohnoe S, Sugimachi K. 5-Fluorouracil is converted to F-nucleotides more extensively and is more cytotoxic in poorly differentiated than in well differentiated human gastric carcinoma. *Anticancer Res* 1990; **10**: 1091–4.
2. Goseki N, Takizawa T, Koike M. Differences in the mode of the extension of gastric cancer classified by histological type: new histological classification of gastric carcinoma. *Gut* 1992; **33**: 606–12.
3. Correa P. The new era of cancer epidemiology. *Cancer Epidemiol Biomarkers Prev* 1991; **1**: 5–11.
4. Semba S, Yokozaki H, Yamamoto S, Yasui W, Tahara E. Microsatellite instability in precancerous lesions and adenocarcinomas of the stomach. *Cancer* 1996; **77**: 1620–7.
5. Yokozaki H, Kuniyasu H, Kitadai Y, Nishimura K, Todo H, Ayhan A, Yasui W, Ito H, Tahara E. p53 point mutations in primary human gastric carcinomas. *J Cancer Res Clin Oncol* 1992; **119**: 67–70.
6. Nakatsuru S, Yanagisawa A, Ichii S, Tahara E, Kato Y, Nakamura Y, Horii A. Somatic mutation of the APC gene in gastric cancer: frequent mutations in very well differentiated adenocarcinoma and signet-ring cell carcinoma. *Hum Mol Genet* 1992; **8**: 559–63.
7. Hattori Y, Itoh H, Uchino S, Hosokawa K, Ochiai A, Ino Y, Ishii H, Sakamoto H, Yamaguchi N, Yanagihara K, Hirohashi S, Sugimura T, Terada M. Immunohistochemical detection of K-sam protein in stomach cancer. *Clin Cancer Res* 1996; **8**: 1373–81.
8. Wong BY, Chen H, Chung SW, Wong PM. High-efficiency identification of genes by functional analysis from a retroviral cDNA expression library. *J Virol* 1994; **68**: 5523–31.
9. O'Dowd BF, Nguyen T, Marchese A, Cheng R, Lynch KR, Heng HH, Kolakowski LF Jr, George SR. Discovery of three novel G-protein-coupled receptor genes. *Genomics* 1998; **47**: 310–3.
10. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
11. Yoshimatsu T, Tamura M, Kuriyama S, Ikenaka K. Improvement of retroviral packaging cell lines by introducing the polyomavirus early region. *Hum Gene Ther* 1998; **9**: 161–72.
12. Ikenaka K, Nakahira K, Nakajima K, Fujimoto I, Kagawa T, Ogawa M, Mikoshiba K. Detection of brain-specific gene expression in brain cells in primary culture: a novel promoter assay based on the use of a retrovirus vector. *New Biol* 1992; **1**: 53–60.
13. Cepko C. Preparation of a specific retrovirus producer cell line. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, editors. Current protocols in molecular biology. Suppl. 17. New York: John Wiley and Sons; 1992. p. 9.11.1–12.
14. Gopalkrishnan RV, Su ZZ, Goldstein NI, Fisher PB. Translational infidelity and human cancer: role of the PTI-1 oncogene. *Int J Biochem Cell Biol* 1999; **31**: 151–62.
15. Foster KW, Ren S, Louro ID, Lobo-Ruppert SM, McKie-Bell P, Grizzle W, Hayes MR, Broker TR, Chow LT, Ruppert JM. Oncogene expression cloning by retroviral transduction of adenovirus E1A–immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. *Cell Growth Differ* 1999; **10**: 423–34.
16. Graness A, Adomeit A, Heinze R, Wetzker R, Liebmann C. A novel mitogenic signaling pathway of bradykinin in the human colon carcinoma cell line SW-480 involves sequential activation of a Gq/11 protein, phosphatidylinositol 3-kinase beta, and protein kinase Cepsilon. *J Biol Chem* 1998; **273**: 32016–22.
17. Antonelli V, Bernasconi F, Wong YH, Vallar L. Activation of B-Raf and regulation of the mitogen-activated protein kinase pathway by the G(o) alpha chain. *Mol Biol Cell* 2000; **11**: 1129–42.
18. Marinissen MJ, Gutkind JS. G-Protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* 2001; **22**: 368–76.
19. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* 2002; **8**: 289–93.
20. Parry MA, Myles T, Tschopp J, Stone SR. Cleavage of the thrombin receptor: identification of potential activators and inactivators. *Biochem J* 1996; **320**: 335–41.
21. Ruol A, Parenti A, Zaninotto G, Merigliano S, Costantini M, Cagol M, Alfieri R, Bonavina L, Peracchia A, Ancona E. Intestinal metaplasia is the probable common precursor of adenocarcinoma in Barrett esophagus and adenocarcinoma of the gastric cardia. *Cancer* 2000; **88**: 2520–8.
22. Kobayashi K, Okamoto T, Takayama S, Akiyama M, Ohno T, Yamada H. Genetic instability in intestinal metaplasia is a frequent event leading to well-differentiated early adenocarcinoma of the stomach. *Eur J Cancer* 2000; **36**: 1113–9.