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Characterization of Gastrin-Releasing Peptide and Its Receptor Aberrantly Expressed by Human Colon Cancer Cell Lines

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ABSTRACT

Gastrin-releasing peptide (GRP) is a mitogen and morphogen important in the development of human colon cancers. Although epithelial cells lining the colon do not normally express GRP or its receptor (GRP-R), most human tumors express GRP-R mRNA. Yet functional protein has only been detected in 24 to 40% of colon cancers. To elucidate the reason for the difference between the expression of GRP/GRP-R mRNA and protein, we studied nine human colon cancer cell lines. Quantitative polymerase chain reaction revealed that all colon cancer cell lines expressed similar amounts of mRNA for both GRP as well as GRP-R. Yet binding studies using ^{125}I-Tyr^{4}-bombesin detected functional receptors on only five of the nine cell lines studied. Conformational fragment-length polymorphism analysis indicated that although mRNA for the ligand GRP was never mutated, mRNA for the GRP-R was always mutated. Sequencing revealed that the message for GRP-R contained between two and seven separate mutations at the nucleotide level. This resulted in 14 separate coding mutations, 2 of which were observed in more than one cell line. Each mutation was individually recreated by site-directed mutagenesis and studied in transiently transfected Chinese hamster ovary-K1 cells. Alteration of Pro^{145} into a tyrosine, of Val^{317} into a glutamic acid, and insertion of a 32-nucleotide segment resulting in a frameshift distal to Asp^{137} all resulted in GRP receptors incapable of binding ligand. Thus, these data indicate that human colon cancers commonly express GRP and GRP-R mRNA but that receptor mutations account for the failure of functional protein to be generated.

Gastrin-releasing peptide (GRP) is a member of the bombesin family of peptide hormones, responsible for multiple diverse effects in the central nervous, immune, pulmonary, and gastrointestinal systems (reviewed in Tache et al., 1988). GRP acts by binding to a specific seven-transmembrane-spanning, G protein-coupled receptor that has been cloned in multiple species including humans (Corgay et al., 1991). Epithelial cells lining the human colon do not normally express GRP or GRP receptors (GRP-R) (Ferris et al., 1997). When aberrantly expressed by colon cancers and cancer cell lines, GRP is well known to act as a mitogen (Frucht et al., 1991, 1992; Radulovic et al., 1991, 1994; Preston and Primrose, 1993; Halmos et al., 1994; Qin et al., 1994). More recently, we have shown that GRP also can act as a morphogen in human colon cancer (Carroll et al., 1999b).

When all studies of GRP-R aberrantly expressed by human colon cancers are reviewed, it is apparent that a higher proportion of tumors express receptor mRNA than functional protein. Whereas as many as 93% of resected human colon cancers express GRP-R mRNA (Saurin et al., 1999), we recently demonstrated, by immunohistochemistry, the presence of protein in only 76% of tumors (Carroll et al., 1999b). And when pharmacological studies have been performed evaluating for the presence of functional receptor, one study identified ^{125}I-Tyr^{4}-bombesin binding sites in only 5 of 21 (24%) tumors (Preston et al., 1995) while another in but 6 of 15 (40%) tumors (Radulovic et al., 1992). The reason(s) for this difference between the expression of RNA and functional protein have not been previously identified.

One of our prior studies suggested that this difference in expression between mRNA and functional protein might be caused by the presence of receptor-inactivating mutations. We recently demonstrated that GRP-R mRNA aberrantly expressed by human gastric adenocarcinomas is frequently mutated, with the mutations having a wide range of pharmacological consequences including receptor inactivation (Carroll et al., 1999a). Given this finding, we hypothesized that mutations resulting in receptor inactivation might explain the discordance between GRP-R mRNA and protein expression in human colon cancer. To evaluate this hypothesis, we studied nine separate human colon cancer cell lines.

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ABBREVIATIONS: GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor; CHO, Chinese hamster ovary; bp, base pair(s); PCR, polymerase chain reaction; CFLP, conformational fragment length polymorphism analysis; RT, reverse transcription; MOPS, 4-morpholinepropanesulfonic acid.

By studying cell lines we could compare the pharmacology of GRP-R expressed by these cells with the mutational profile of this receptor's mRNA. All mutations identified were recreated and evaluated pharmacologically in transiently transfected Chinese hamster ovary (CHO)-K1 cells, which we have previously shown to be a good model for the study of GRP-R expressed by nonmalignant cells (Carroll et al., 1999a). We herein demonstrate that all human colon cancer cell lines express similar amounts of GRP and GRP-R mRNA; that the mRNA for GRP is never mutated, whereas that for the GRP-R is always mutated; and that these mutations frequently result in receptor inactivation. These findings identify a hitherto unappreciated mechanism responsible for determining whether a mitogen and morphogen important to the growth and differentiation of colon cancer is expressed as a functional protein.

Experimental Procedures

Materials. *Taq* polymerase was obtained from Perkin-Elmer (Foster City, CA) and *Pfu* polymerase was from Stratagene (La Jolla, CA), and Cleavase was from Gibco/BRL (Gaithersburg, MD). All restriction enzymes were from Promega (Madison, WI). Cloning vectors including pCR2.1 and pcDNA-3 were from InVitrogen (Carlsbad, CA). All DNA purifications were performed using GeneClean III (Bio 101, Vista, CA). All radionucleotides were obtained from Amersham (Arlington Heights, IL). All reagents not otherwise specified were molecular grade from Sigma Chemical (St. Louis, MO).

Quantitative Polymerase Chain Reaction (PCR). mRNA for both GRP and GRP-R were determined using a mimic. Human GRP and GRP-R cDNA were obtained from Dr. James Battey (National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD), and subcloned in pCR2.1. A GRP mimic was created by deleting a 253 base-pair (bp) region (from +60 to +313, ATG = +1) from the coding region of the GRP cDNA using *Sma*I/*Stu*I. Likewise a GRP-R mimic was created by deleting a 287-bp region (from +161 to +448; ATG = +1) from the coding region of the GRP-R cDNA using *Stu*I alone. The modified mimic cDNAs were excised from their vectors and quantified.

mRNA from each cell line was extracted and subjected to reverse transcription. Fixed amounts of cDNA were then spiked with known amounts of mimic cDNA. Amplification was performed using gene-specific primers for GRP (forward, 5′-AGT CTC TGC TCT TCC AGC C-3′; reverse, 5′-CCG ATG GAC AAC CAA TCT AAG-33′) and GRP-R (forward, 5′-CAT GCA CTG CAA CAT CTC C-3′; reverse, 5′-GAC CAG AAA GGA AGC CAT A-3′). The following conditions were used: hot start; six cycles of 94°C for 15 s and 72°C for 30 s, followed by 24 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s. PCR for GRP generated a product of 503 bp for the mimic and 753 for the native message, whereas that for the GRP-R generated a product of 300 bp for the mimic and 587 bp for the native message. Products were resolved electrophoretically on a 1% agarose gel (as shown in the inset of Fig. 1) and images acquired using an Eagle Eye Detection System (Stratagene).

Mutation Identification. Tumor cell RNA was cloned using the gene-specific primers A (forward, 5′-GGA GAC TCA GAC TAG AAT GG-3′) and D (reverse, 5′- CCA GTG CTG TGA GAC CGG AT-3′) for the GRP-R, or the primers described above for GRP. To minimize polymerase errors, PCR reactions were performed in the presence of a 16:1 mixture of *Taq*:*Pfu* containing 2.5 mM $MgCl_2$, 6% dimethyl sulfoxide, and 3% glycerol, as described previously (Carroll et al., 1999a). The whole length GRP or GRP-R PCR product was subcloned into pCR2.1, and a minimum of four separate clones were amplified and then subjected to both conformational fragment-length polymorphism analysis (CFLP) analysis and dideoxy sequencing (Sanger et al., 1977).

We rapidly screened for the presence of mutations in the coding sequence for both GRP and GRPR by conformational fragment-length polymorphism analysis (CFLP) using a modification of the method of Lyamichev et al. (1993) and Brow et al. (1996) as described previously (Carroll et al., 1999a). Because of the length of the mRNA for GRP-R, we were able to screen for mutations only after performing nested PCR. Briefly, reverse transcription (RT)-PCR using forward primer A and reverse primer B (5′-ACA TAC CGG TCG TGA CAG AT-3′) was performed against RNA obtained for all cell lines. In separate nested reactions, forward primer C (5′-AGC CAG AGC AGC ACC AGT GT-3′) was end-labeled using [γ-^{32}P]ATP and combined with unlabeled reverse primer D. In the other reaction, however, end-labeled primer D was combined with unlabeled primer C. This approach allowed us to analyze PCR product focusing on either the 5′ or 3′ regions of the GRP-R. In both cases, labeled primer was used in 5-fold excess of unlabeled primers in a 30-cycle PCR reaction (denature, 94°C for 15 s; anneal, 52°C for 30 s; extension, 72°C for 60 s). PCR reaction products were purified using GeneClean III, diluted to 10^5 cpm/13 μl in milliQ-purified water, and the entire sample was placed at 95°C for 2 min. Samples were then placed for 30 s at 50°C when using labeled primer C, or at 55°C when using labeled primer D (temperature optimization data not shown). The reaction products were digested with 25 U of Cleavase (Gibco/BRL) in 28.5 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.5, and 2 mM $MnCl_2$ at the same temperature for an additional 2 min. The reaction was quenched with 16 μl of 10 mM EDTA, pH 8.0, in 95% formamide. Reaction products were resolved on a 6% polyacrylamide gel, and products identified after exposure using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The identification of mutations in the mRNA for the ligand GRP was performed similarly, except that only a single round of PCR was necessary given the smaller size of the message. RT-PCR was performed against the full-length RNA obtained for all cell lines using

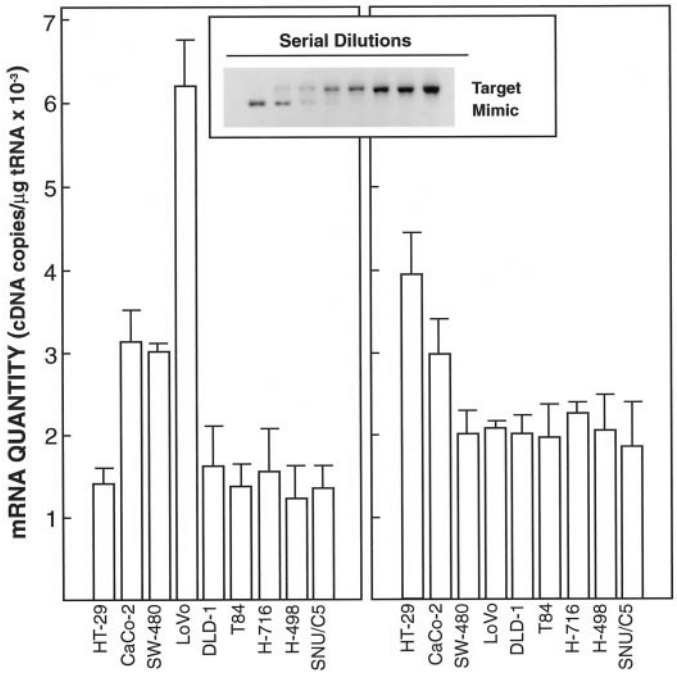

Fig. 1. Quantitative PCR showing the amount of GPR (left) and GRP-R (right) mRNA present in human colon cancer cell lines. mRNA from the indicated cell lines was subjected to RT and then amplified by PCR in the presence of varying known quantities of mimic as described under *Experimental Procedures*. Lanes containing similar amounts of target and mimic message (inset, lane 3 from left) identified the quantity of mRNA present in the cell line, expressed as cDNA copies per microgram of tRNA extracted. Data are expressed as the mean ± S.E. for a minimum of three separate experiments.

forward primer E (5′-AGT CTC TGC TCT TCC AGC C-3′) that was end-labeled using [γ-^{32}P]ATP, and reverse primer F (5′-CCG ATG GAC AAC CAA TCT AAG-3′). The PCR products were then treated identically as described for the GRP-R reaction product.

In all instances, identical CFLP patterns for GRP and GRP-R were obtained for all clones evaluated per cell line. To specifically identify the individual mutations present in each cell line, dideoxy (Sanger) sequencing of the entire GRP and GRP-R coding sequence was performed on at least four separate clones containing the RT-PCR products within vector pCR2.1

Site-Directed Mutagenesis. To study the pharmacological consequences of the reported mutations, each was recreated separately by site-directed mutagenesis and studied in transiently transfected CHO-K1 cells. Sense and antisense mutagenic primers (27-mers) were designed for each missense mutation identified using the QuikChange Site-Directed Mutagenesis System (Stratagene). For each reaction, 125 ng of appropriate forward and reverse mutagenic primers were combined with 50 ng of wild-type human GRP-R cDNA in pcDNA-3, 10 nM dNTP, 2 mM $MgSO_4$ in 20 mM Tris·HCl, pH 8.8, 3% glycerol, 6% dimethyl sulfoxide, and 2.5 U of Pfu DNA polymerase in a 12-cycle PCR reaction (denature, 95°C, 30 s; anneal, 55°C, 60 s; extension, 68°C, 12 min). Original methylated plasmid DNA was destroyed by digesting with 10 U of *Dpn*I, and the newly synthesized DNA was transformed into Epicurean Coli XL-1 Blue cells. All plasmids were completely sequenced (Sanger et al., 1977) to confirm the presence of the desired mutation and rule out the formation of spurious polymerase-induced mutations.

Cell Culture and Transfection. Wild-type and mutant GRP-R in pcDNA-3 were studied in transiently transfected CHO-K1 cells as described previously (Carroll et al., 1999a). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and maintained in a humidified chamber containing 5% CO_2 at 37°C. When cells were ~30% confluent, transient transfection was performed using LipofectAMINE (Sigma) according to the manufacturer's instructions. Cells were studied 48 h post-transfection.

Binding Assays. Binding studies were performed after mechanically disaggregating washed cells, which were then resuspended in 50 mM Tris·HCl, pH 7.5, containing 0.2% BSA, and 0.1% bacitracin, pH 7.5 (Benya et al., 1995; Ferris et al., 1997). In all instances, binding reactions were performed in the presence of 75 pM ^{125}I-Tyr^4-bombesin and performed using 5×10^6 cells/ml for 60 min at 22°C. Nonsaturable binding was the amount of cell-associated radioactivity when the incubation mixture contained 1 μM bombesin, with nonsaturable binding being <15% of total binding in all experiments. All values are reported as saturable binding (i.e., total minus nonsaturable binding).

Results

Expression of GRP-R Binding Sites. We first determined if the nine cell lines expressed GRP-R binding sites. Overall, in only five of the nine human colon cancer cell lines was the GRP analog bombesin able to specifically inhibit binding of ^{125}I-Tyr^4-bombesin (Table 1). Specifically, bombesin inhibited ^{125}I-Tyr^4-bombesin binding to CaCo-2, H-716, HT-29, LoVo-E2, and SW-480 cells with approximately equal ability, with K_i values ranging from ~1.5 to 2.5 nM. Scatchard analysis of the binding data performed using the least-squares curve-fitting program Ligand (Muson and Robard, 1980) indicated that the binding data was best fit by a single-site model in all instances. Overall, similar numbers of GRP-R were detected on these cell lines, with 5,700 ± 400 binding sites/cell on CaCo-2 cells; 3,100 ± 500 on H-716 cells; 4,700 ± 300 on HT-29 cells; 11,400 ± 800 on LoVo-E2 cells; and 5,100 ± 300 on SW-480 cells. These binding affinities and receptor numbers are similar to what have previously been described for HT-29 cells (Radulovic et al., 1991; Radulovic et al., 1994), LoVo-E2 (Saurin et al., 1999), and H-716 cells (Frucht et al., 1991; Frucht et al., 1992), indicating that significant clonal variation did not exist between the same cell lines studied in this article and as reported in previous investigations. We next determined if this pharmacological difference was caused by differences in mRNA expression between the various cell lines.

GRP/GRP-R mRNA Is Ubiquitously Expressed by Human Colon Cancers. To determine the amount of mRNA present in each cell line, we performed quantitative PCR. All cell lines expressed RNA for both GRP as well as GRP-R, with similar quantities for both detected in all (Fig. 1). GRP mRNA expression varied ~6-fold across the cell lines evaluated. Specifically, the amount of GRP mRNA ranged from a low in H-489 cells (1100 ± 300 cDNA copies/μg of tRNA) to a high in HT-29 cells (6500 ± 600 cDNA copies/μg of tRNA). In contrast, the amount of GRP-R mRNA was fairly similar across all cell lines such that there was only 2-fold more mRNA in HT-29 cells, and 1.5-fold more in CaCo-2 cells, than detected in the other cell lines (Fig. 1). Because all cell lines expressed GRP-R mRNA but functional protein was detected in only five cell lines, this suggested the possibility that receptor-inactivating mutations might be present.

Mutational Analysis. To rapidly screen for the presence of mutations in aberrantly expressed GRP/GRP-R mRNA, we performed CFLP. In all cases, CFLP was performed in RNA extracted from BALB 3T3 cells stably expressing nonmutated human GRP or GRP-R (Benya et al., 1995) that was used for comparative purposes. Comparing what has been described as a "bar code fingerprint" (Brow et al., 1996) generated by wild-type GRP and GRP-R, it can be readily appreciated that message for ligand was never mutated, whereas that for receptor was always mutated (Fig. 2). This finding was confirmed by Sanger sequencing (Sanger et al., 1977). To minimize the development of polymerase-induced errors, we performed RT-PCR using a combination of *Taq*/*Pfu* in the presence of dimethyl sulfoxide and glycerol. We then sequenced the entire GRP and GRP-R sequence contained in a minimum of four separate clones resulting as a consequence of each RT-PCR reaction. In all instances, sequencing revealed the presence of the same mutations in all clones.

Between two and seven separate nucleotide mutations were identified in each colon cancer cell line studied, with six of the mutations identified in more than one cell line (Table 1). Overall, 23 different nucleotide mutations were identified, 9 of which were silent and 14 of which resulted in altered amino acid sequences. Of these amino acid-altering mutations, two (P145Y and F178L) were detected in more than one cell line. Of these, F178L has also been previously identified to occur in GRP-R mRNA aberrantly expressed by human gastric adenocarcinomas (Carroll et al., 1999a).

Impact of the Identified Mutations on GRP-R Pharmacology. Each of the mutations identified in Table 1 was recreated by site-directed mutagenesis and studied in transiently transfected CHO-K1 cells to determine their effect on GRP-R pharmacology. A total of 14 separate mutants were evaluated. In all instances, 10 μg of wild-type or mutant GRP-R cDNA in vector pcDNA-3 resulted in similar numbers of expressed receptors, except for the three mutants incapable of binding ^{125}I-Tyr^4-bombesin (Table 2). The binding af-

finities of the remaining 11 mutants were similar (~1–2 nM) to that which we have previously described for nonmutated wild-type human GRP-R expressed by a variety of cell types (Benya et al., 1995; Ferris et al., 1997; Carroll et al., 1999a). In contrast, substituting the proline at amino acid position 145 with a tyrosine, replacing the valine at amino acid position 317 with a glutamic acid, or truncating the receptor after the aspartic acid at position 137 resulted in GRP-R incapable of binding agonist.

Discussion

We observed previously that the pharmacological profile of GRP-R aberrantly expressed by gastrointestinal cancers and cancer cell lines differs significantly from that observed in nonmalignant cells (Ferris et al., 1997). Specifically, GRP-R expressed by nonmalignant cells binds agonist in the nanomolar range, whereas Scatchard analysis of the binding data is best described by a one-site model (Ferris et al., 1997). In contrast, many but not all GRP-R aberrantly expressed by cancers of the gastrointestinal tract bind agonist in the picomolar range and are not infrequently described by a two-site model. This prompted us to postulate that the alterations in pharmacological profile might be caused by the GRP-R being mutated when aberrantly expressed by gastrointestinal cancers.

Additional evidence for the presence of receptor mutations, particularly those resulting in receptor inactivation, is suggested by the gross discrepancy between GRP-R mRNA and protein expression. A study of 29 resected human colon cancers found that 27 (93%) aberrantly expressed GRP-R mRNA

TABLE 1

GRP-R mRNA nucleotide and amino acid mutations identified in human colon cancer cell lines with binding inhibition constants as determined against natively expressed receptors

Nucleotides and amino acids in single-letter code. Nucleotide numbering is from the site of translation initiation, whereas that for amino acid numbering is from the site of translation initiation (i.e., ATG = +1). Entries preceded by a bullet point identify mutations seen in more than one cell line. Binding data is for a minimum of three separate transfections as described under *Experimental Procedures*, with data presented as means ± S.E.

Cell Line	Mutations				K_i
	Nucleotide	Amino Acid	Location	Other Lines?	
					nM
CaCo-2	C455T	Silent	NH_2		1.5 ± 0.3
	•C851T	Silent	IC-2	DLD-1 HT-29	
	•C1061T	Silent	TM-5	DLD-1 HT-29 T-84	
DLD-1	C504A	H36N	NH_2		N.B.
	T539C	Silent	TM-1		
	A818G	Silent	IC-2		
	•C851T	Silent	IC-2	CaCo-2 HT-29	
	A1023G	I209V	EC-3		
	•C1061T	Silent	TM-5	CaCo-2 HT-29 T-84	
	•T1348A	V317E	TM-7		
H-498	A498G	N31D	NH_2		N.B.
	A513G	V40I	TM-1		
	Insertion[a]	Stop Codon	IC-2		
H-716	T854C	H152Q	IC-2		2.3 ± 0.7
	A1005G	N203S	EC-2		
HT-29	C695T	Silent	EC-2		1.9 ± 0.4
	•C851T	Silent	IC-2	CaCo-2 DLD-1	
	•C1061T	Silent	TM-5	CaCo-2 DLD-1	
LoVo-E2	•C926T	Silent	TM-4	SW-480	1.5 ± 0.2
	•T930C	F178L	EC-2	SW-480	
	T1057C	V220A	TM-5		
SNU/C5	•C831A	P145Y	IC-2	T-84	N.B.
	C881A	Silent	TM-4		
SW-480	A496T	D33V	NH_2		2.0 ± 0.3
	•C926T	Silent	TM-4	LoVo-E2	
	•T930C	F178L	EC-2	LoVo-E2	
T-84	•C831A	P145Y	IC-2	SNU/C5	N.B.
	T862C	M155Y	TM-4		
	•C1061T	Silent	TM-5	CaCo-2 DLD-1 HT-29	
	T1068C	S224Y	TM-5		
	T1108C	Silent	IC-3		

N.B., no specific binding to the cell line observed.

[a] Insertion of a 32-nucleotide segment resulting in a frameshift and ultimately a stop codon.

(Saurin et al., 1999). Yet prior pharmacological studies identified ^{125}I-Tyr^4-bombesin binding sites on membranes of only 24% (Preston et al., 1995) to 40% (Radulovic et al., 1992) of human colon cancers. In contrast, our immunohistochemical study of GRP and GRP-R expression by human colon cancers (Carroll et al., 1999b) indicated that many more receptors were being expressed than had been previously detected pharmacologically. Specifically, we found evidence for GRP-R expression by immunohistochemistry in 38 of 50 (76%) randomly selected colon cancers, irrespective of tumor stage (Carroll et al., 1999b).

In the current study, we found that all colon cancer cell lines aberrantly expressed GRP/GRP-R mRNA (Fig. 1), but that specific binding of ^{125}I-Tyr^4-bombesin was only detected in five of nine cell lines (Table 1). In all cell lines not capable of binding ^{125}I-Tyr^4-bombesin, GRP-R mRNA mutations resulting in nonfunctional receptor were detected (Table 2). Thus our data suggests that GRP/GRP-R mRNA may be frequently, if not ubiquitously, expressed by human colon cancers and cancer cell lines; but that message for receptor, at least, is also frequently mutated, with many mutations rendering the GRP-R incapable of binding agonist.

GRP-R mutations are not restricted to cancers of the colon. We found previously that human gastric cancers also express GRP-R that are commonly mutated (Carroll et al., 1999a). In that study, we found GRP-R mRNA to be aberrantly expressed by 8 of 20 endoscopically biopsied gastric cancers. Of these, six contained one or more mutations, with two of these causing GRP-R inactivation. Interestingly, one of the same mutations reported therein was also identified in our current study of colon cancer cell

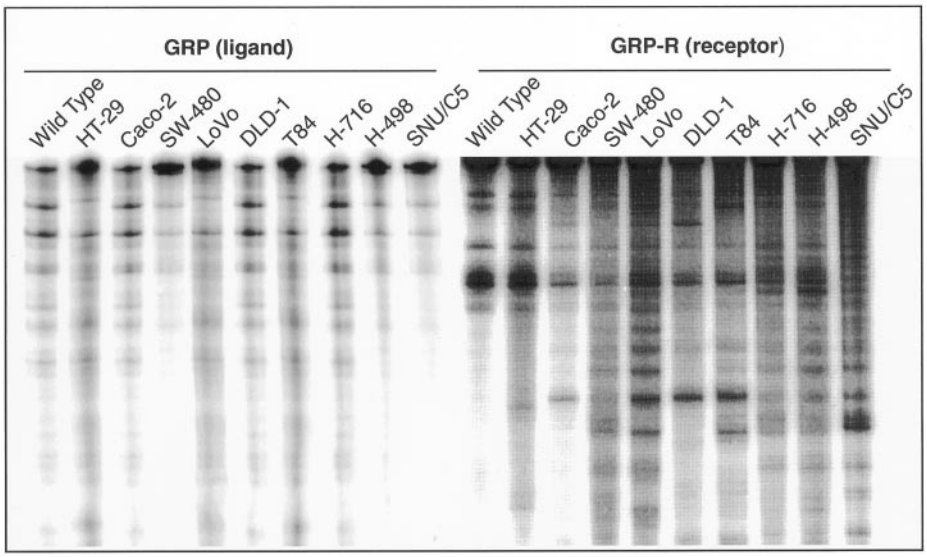

Fig. 2. CFLP analysis of GRP (left) and GRP-R (right) mRNA expressed by the indicated cell lines. Wild-type mRNA was obtained from Balb 3T3 fibroblasts stably transfected with plasmid pcDNA-3 containing the nonmutated cDNA for the human GRP or GRP-R sequence. RNA was subject to RT-PCR and CFLP performed as described under *Experimental Procedures*. Appreciate the similarity in the "bar code fingerprint" for GRP across cell lines indicating the absence of mutations. In contrast, that for GRP-R is completely different in all cell lines and from that of wild-type message, indicating the presence of mutations.

TABLE 2

Effect of the indicated mutations on GRP-R pharmacology when transiently expressed in CHO-K1 cells

Mutant constructs are identified by single-letter amino acid code. Data are expressed as means ± S.E. for a minimum of three separate transfections.

Mutation	Location	Cell Lines	Receptor Pharmacology	
			K_i	B_{max}
			nM	*sites/cell*
Wild type	Not relevant	CHO-K1	1.4 ± 0.2	18,400 ± 700
N31D	NH_2	H-498	2.1 ± 0.4	21,600 ± 800
D33V	NH_2	SW-480	1.9 ± 0.5	17,900 ± 800
H36N	NH_2	DLD-1	2.2 ± 0.4	14,200 ± 500
V40I	TM-1	H-498	1.6 ± 0.3	16,900 ± 700
P145Y	IC-2	SNU/C5	N.B.	N.B.
		T-84		
H152Q	IC-2	H-716	2.2 ± 0.5	18,200 ± 700
M155Y	TM-4	T-84	1.7 ± 0.4	25,000 ± 1,400
F178L	EC-2	LoVo-E2	1.6 ± 0.4	21,700 ± 1,900
		SW-480		
N203S	EC-2	H-716	2.4 ± 0.3	19,900 ± 700
I209V	EC-2	DLD-1	1.7 ± 0.6	20,300 ± 1,100
V220A	TM-5	LoVo-E2	0.8 ± 0.5	21,400 ± 1,200
S224Y	TM-5	T-84	1.4 ± 0.3	17,600 ± 300
V317E	TM-7	DLD-1	N.B.	N.B.
Insertion/deletion	IC-2	H-498	N.B.	N.B.

N.B., no specific binding to the cell line observed.

lines [F178L, mislabeled as F177L in (Carroll et al., 1999a)]. Thus, these findings suggest that GRP-R mutations are common in cancers of the gastrointestinal tract, with amino acid mutations distributed diffusely throughout the coding region of this receptor (Fig. 3). Our findings also indicate that multiple different regions of the GRP-R are important for mediating agonist binding. Unsurprisingly, the proline substitutions detected both in this study (P145Y) and in our study of gastric cancers (P182L, P199S) (Carroll et al., 1999a) resulted in the production of pharmacologically nonfunctional receptors. More intriguing is the identification of V317E as disruptive to agonist binding. Val^{317} is immediately adjacent to the putative internalization consensus sequence $NP(X)_nY$. Although alterations of this sequence have not been shown to alter internalization of the murine GRP-R (Slice et al., 1994), the effect of mutations in this regions indicate that it nonetheless remains important to overall receptor function.

This study did not attempt to determine whether the mutations in GRP-R mRNA occurred in any particular allele of the *GRPR* gene. It could be argued that our identification of multiple different mutations in any particular cell line might represent single mutations present in different alleles, particularly because dramatic karyotypic changes in cancer cell lines are known to result in allelic expansion and contraction. In this article, we studied only mRNA mutations and thus limited our investigations to the product of active allele(s). Furthermore, we fully sequenced the GRP and GRP-R PCR products contained in at least four separate clones that had been generated from each RT-PCR reaction. Because RNA was extracted from each cell line on three separate occasions and independently subjected to RT-PCR, GRP and GRP-R mRNA was sequenced at least 12 different times. In all instances, the same multiple mutations were identified in all clones. Thus this strongly suggests that the active allele(s) contained all the mutations identified per cell line.

In aggregate, our findings indicate that the GRP-R is completely different from other heptaspanning receptors that are mutated in various disease states. Whereas *constitutively activating* mutations in heptaspanning receptors have been implicated for diseases ranging from thyroid adenomas [thyroid stimulating hormone receptor (Paschke and Ludgate, 1997)] to precocious puberty [luteinizing hormone receptor (Shenker et al., 1993; Kosugi et al., 1995)], we are the first to identify the possible importance of *inactivating* mutations. We recently showed that colon cancers developing in mice genetically incapable of synthesizing GRP-R (i.e., $GRP\text{-}R^{-/-}$ mice) progressively dedifferentiate over time, whereas tumors in wild-type mice expressing this receptor become increasingly better differentiated (Carroll et al., 2000). This study conclusively demonstrated the important contribution of the GRP-R to colon cancer development and progression. In concert with the present study, our data support the novel hypothesis that colon cancer dedifferentiation may in part be regulated by the accumulation of GRP-R-inactivating mutations.

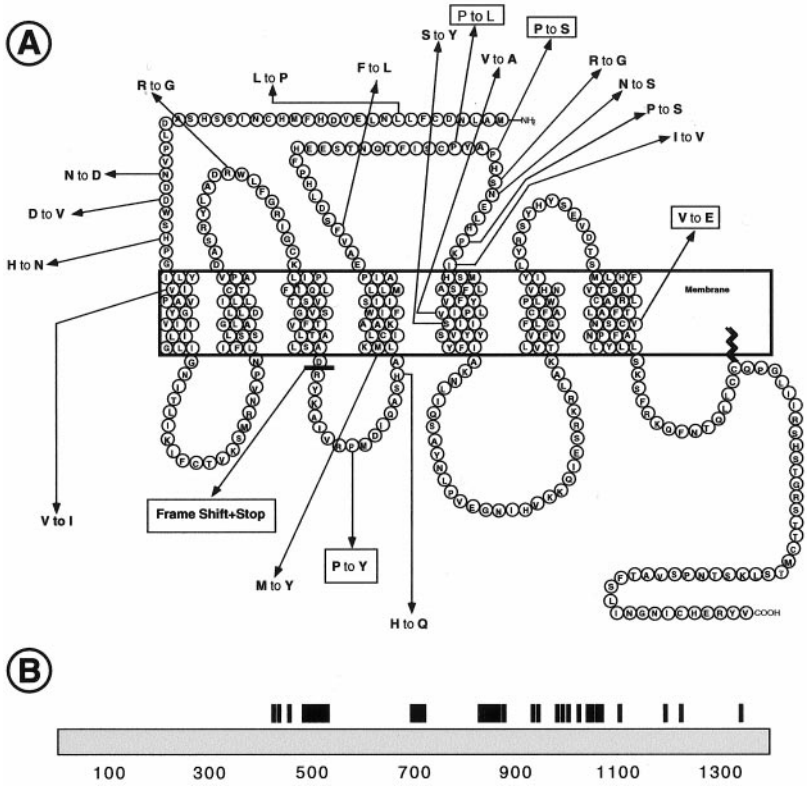

Fig. 3. Presence of all amino acid-altering mutations identified to date in the GRP-R when aberrantly expressed by cancers of the stomach and colon. A, secondary structure of the human GRP-R identifying the individual amino acids mutated. Boxed mutations indicate those that result in the GRP-R being unable to bind ligand. B, linear representation of the *GRP-R* gene with hash marks identifying the location of all mutations including those that are silent.

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