3. E. Heinz and P. G. Roughan, *Plant Physiol.* **72**, 273 (1983).
4. S. Mongrand, J.-J. Bessoule, F. Cabantous, C. Cassagne, *Phytochemistry* **49**, 1049 (1998).
5. L. Kunst, J. Browse, C. R. Somerville, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4143 (1988).
6. J. Browse and C. R. Somerville, in *Arabidopsis*, E. M. Meyerowitz and C. R. Somerville, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994), pp. 881–912.
7. P. Dörmann, S. Hoffmann-Benning, I. Balbo, C. Benning, *Plant Cell* **7**, 1801 (1995).
8. H. Härtel, H. Lokstein, P. Dörmann, B. Grimm, C. Benning, *Plant Physiol.* **115**, 1175 (1997); F. Reifarth *et al.*, *Biochemistry* **36**, 11769 (1997); H. Härtel, H. Lokstein, P. Dörmann, R. N. Trethewey, C. Benning, *Plant Physiol. Biochem.* **36**, 407 (1998); L.-J. Chen and H.-M. Li, *Plant J.* **16**, 33 (1998).
9. A. van Besouw and J. F. G. M. Wintermans, *Biochim. Biophys. Acta* **529**, 44 (1978); J. W. M. Heemskerk, T. Storz, R. R. Schmidt, E. Heinz, *Plant Physiol.* **93**, 1286 (1990).
10. J. Browse, N. Warwick, C. R. Somerville, C. R. Slack, *Biochem. J.* **235**, 25 (1986).
11. An F_2 population of plants derived from the cross of *act1* (*Arabidopsis* Biological Resource Center, Columbus, OH) and *dgd1* was screened by TLC or gas chromatography. About 1/16th of the F_2 plants were homozygous *act1*,*dgd1* double mutants with reduced amounts of 7,10,13-hexadecatrienoic acid as in *act1* and reduced amounts of the digalactosyl lipid as in *dgd1*.
12. M. Shimojima *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 333 (1997).
13. M. A. Block, A.-J. Dorne, J. Joyard, R. Douce, *J. Biol. Chem.* **258**, 13281 (1983).
14. K. Cline and K. Keegstra, *Plant Physiol.* **71**, 366 (1983).
15. T. Teucher and E. Heinz, *Planta* **184**, 319 (1991); E. Maréchal, M. A. Block, J. Joyard, R. Douce, *J. Biol. Chem.* **269**, 5788 (1994).
16. Of 135 F_2 plants derived from the cross *dgd1* × Col-2, plants with crossovers between markers nga162 and nga172 were selected [C. J. Bell and J. R. Ecker, *Genomics* **19**, 137 (1994)]. In this F_2 population, we mapped the *DGD1* locus relative to markers g4523, *fad7*, g4547, 5E-5, and 18A-1. Similarly, we screened 424 F_2 plants from the cross *dgd1* × *Ler* for crossovers between markers nga127 and ATHCHIB. We used this mapping population to score markers g2488a and 31A-H. We obtained the RFLP markers from the *Arabidopsis* Biological Resource Center (g4523, *fad7*, g2488a, g4547) or from genomic fragments (31A-H, 5E-5, 18A-1) isolated from cosmids.
17. We used DNA of *fad7*, g4547, and g2488a to isolate genomic DNA fragments from different libraries {CIC yeast artificial chromosome library [C. Camilleri *et al.*, *Plant J.* **14**, 633 (1998)], IGF bacterial artificial chromosome library [T. Mozo, S. Fischer, H. Shizuya, T. Altmann, *Mol. Gen. Genet.* **258**, 562 (1998)], and *Arabidopsis* cosmid library [K. Meyer, G. Benning, E. Grill, in *Genome Mapping in Plants*, A. H. Patterns, Ed. (Academic Press, New York, 1996), pp. 137–154]}.
18. Cosmid clones were transferred into *Agrobacterium tumefaciens* C58C1(pGV2260) and used to transform *Arabidopsis* Col-2 wild type by vacuum infiltration [N. Bechtold, J. Ellis, G. Pelletier, *C. R. Acad. Sci. Ser. III Life Sci.* **316**, 1194 (1993); A. F. Bent *et al.*, *Science* **265**, 1856 (1994)]. We crossed transformants with *dgd1* plants and analyzed segregation in the F_2 generation. Complementation was assumed when of a minimum of 100 tested F_2 plants (one to three independent crosses per cosmid) carrying the T-DNA, all were phenotypically wild type.
19. U. Uwer, L. Willmitzer, T. Altmann, *Plant Cell* **10**, 1277 (1998).
20. For direct complementation analysis, the *DGD1* cDNA released from pBluescriptIISK(+) with Sma I, Xho I was ligated into Sma I, Sal I of pBINAR-Hyg [D. Becker, *Nucleic Acids Res.* **18**, 203 (1990); A. von Schaeven, thesis, Freie Universität Berlin (1989)] in the sense orientation behind the CaMV 35S promoter. This construct was directly transferred into *dgd1*.
21. A 459-bp Xho I, Pvu II fragment including the expression cassette was isolated from pQE31 (Qiagen Inc.) and ligated into the Sal I, Eco RV sites of pACYC184 [A. C. Y. Chang and S. N. Cohen, *J. Bacteriol.* **134**, 1141 (1978)], giving rise to the plasmid pACYC-31. We amplified the open reading frame of the *DGD1* cDNA by polymerase chain reaction with the primers (5′-GCGGATCCGGTAAAGGAAACTCTAATT) and (5′-TTCTGCAGTCTACCAGCCGAAGATTGG), thereby introducing a Bam HI site at the 5′ terminus and a Pst I site at the 3′ terminus. We ligated this cDNA fragment (Bam HI/Pst I) into pACYC-31. The resulting plasmid pACYC-31/239 was transferred into XL1-Blue cells containing the expression vector pGEX-3X with the cucumber monogalactosyl lipid synthase cDNA (*12*).
22. A.-J. Dorne, M. A. Block, J. Joyard, R. Douce, *FEBS Lett.* **145**, 30 (1982).

23. Supported in part by grants from the US Department of Energy and from the Bundesministerium für Bildung und Forschung to C.B. and by a Feodor Lynen fellowship from the Alexander von Humboldt-Foundation and a research scholarship from the Deutsche Forschungsgemeinschaft to P.D. We are indebted to Ed Tolbert for stimulating discussions up until his death in December 1998. We thank H. Ohta for providing the monogalactosyl lipid synthase plasmid. We are grateful to B. Hintzmann and A. Rosenthal for sequencing genomic DNA and cDNA clones. The help of J. Huber in screening and analyzing complemented plants is especially acknowledged.

10 March 1999; accepted 19 May 1999

Receptor for Motilin Identified in the Human Gastrointestinal System

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Motilin is a 22–amino acid peptide hormone expressed throughout the gastrointestinal (GI) tract of humans and other species. It affects gastric motility by stimulating interdigestive antrum and duodenal contractions. A heterotrimeric guanosine triphosphate–binding protein (G protein)–coupled receptor for motilin was isolated from human stomach, and its amino acid sequence was found to be 52 percent identical to the human receptor for growth hormone secretagogues. The macrolide antibiotic erythromycin also interacted with the cloned motilin receptor, providing a molecular basis for its effects on the human GI tract. The motilin receptor is expressed in enteric neurons of the human duodenum and colon. Development of motilin receptor agonists and antagonists may be useful in the treatment of multiple disorders of GI motility.

Gastrointestinal motility is a coordinated neuromuscular process that transports nutrients through the digestive system (*1*). Impaired GI motility, which can lead to gastroesophageal reflux disease, gastroparesis (diabetic and postsurgical), irritable bowel syndrome, and constipation, is one of the largest health care burdens of industrialized nations. Motilin, a peptide that is secreted by enterochromaffin cells of the small intestine (*2*), exerts a profound effect on gastric motility by inducing interdigestive (phase III) antrum and duodenal contractions (*3–5*). The structurally unrelated macrolide antibiotic erythromycin induces similar effects that are perhaps mediated by interaction with motilin receptors in the GI tract, accounting for erythromycin's side-effects, including vomiting, nausea, diarrhea, and abdominal muscular discomfort (*6, 7*).

Motilin is highly conserved across species and is synthesized as part of a larger inactive prohormone. Mature motilin (22 amino acids) is generated by removal of its secretory signal peptide and cleavage in its COOH-terminus (*8–11*). Although high-affinity binding sites for motilin have been detected in the GI tract of humans and other species and in the central nervous system of rabbits, their molecular structure has remained undefined (*12–18*). High-affinity (dissociation constant, $K_d \sim 2$ nM) and low-density (binding capacity, $B_{max} \sim$

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20 fmol per milligram of protein) binding sites for motilin are present in smooth muscle cells of the GI tract of humans, cats, and rabbits (*12–17*) and in rabbit cerebellum (*18*).

G protein–coupled receptors (GPC-Rs) related to the hypothalamic and pituitary receptor for the growth hormone secretagogues (GHSs) (*19*) were cloned from human genomic DNA libraries (*20*). GHSs mediate sustained pulsatile growth hormone release (*21*). One of these clones, GPR38, showed a 52% overall amino acid sequence identity to the human GHS receptor (GHS-R), with 86% sequence identity in the transmembrane (TM) domains. GPR38 was classified as an orphan GPC-R because its natural ligand had not yet been identified. Expression of RNA encoding GPR38 was found in human stomach, thyroid, and bone marrow. The GPR38 protein sequence was predicted solely on the basis of genomic DNA sequence because efforts to isolate cDNA clones by standard library screening proved unsuccessful and exon-intron boundaries could not be resolved (*20*).

Here, we isolated cDNA clones representing correctly spliced GPR38 mRNA using a combination of techniques. Rapid amplification of cDNA ends (RACE) and reverse transcriptase polymerase chain reaction (RT-PCR) were conducted on RNA isolated from human thyroid and from cells transfected with an expression plasmid containing the GPR38 intron. Two distinct sequences were revealed that correspond to alternative use of two splice-donor sequences and a common splice-acceptor sequence (*22*). GPR38-A mRNA encoded a polypeptide of 412 amino acids with seven predicted α-helical TM domains, the hallmark feature of GPC-Rs (*23*), whereas GPR38-B mRNA encoded a 386–amino acid polypeptide with five predicted TM domains (Fig. 1). GPR38 was shown to be a single conserved gene in mammals (*20*) and map to human chromosome 13q14-q21, though defects in GI function that map to this region have not been reported. To determine if the GPR38 gene is conserved distantly in evolution, we identified a related gene (75E7) in the teleost puffer fish *Spheroides nephelus* (which evolved ~400 million years ago), a species that contains ~90% of all mammalian genes (*24*). The protein sequence for 75E7 was 54% identical to human GPR38 (47% to the human GHS-R), contained a similar exon-intron structure, and therefore may represent an ortholog of the human GPR38 (*25*).

GHS-Rs and native receptors for motilin appear to be GPC-Rs that activate the phospholipase C signal transduction pathway (*16, 21*). Hence, to identify a ligand for GPR38-A that contains seven TM domains, we developed a high-throughput assay that measures Ca^{2+} release with aequorin, a bioluminescent Ca^{2+}-sensitive reporter protein. In this assay, activation of a GPC-R by a ligand induces inositol trisphosphate–coupled mobilization of intracellular calcium and concomitant calcium-induced aequorin bioluminescence (*26–28*). A broad set of peptide and nonpeptide molecules was tested at a single concentration (100 nM peptide, 1 μM nonpeptide) in a stable cell line expressing aequorin (HEK-293/aeq17) in the presence and absence of GPR38-A protein that was expressed transiently (*28*). GPR38-A was expressed as a single protein species of relative molecular mass ~45,000 (*29*). These sets (>500 substances) contained ligands for known and unknown membrane receptors, ion channels, and intracellular signaling enzymes and receptors (*30*). A bioluminescent response that

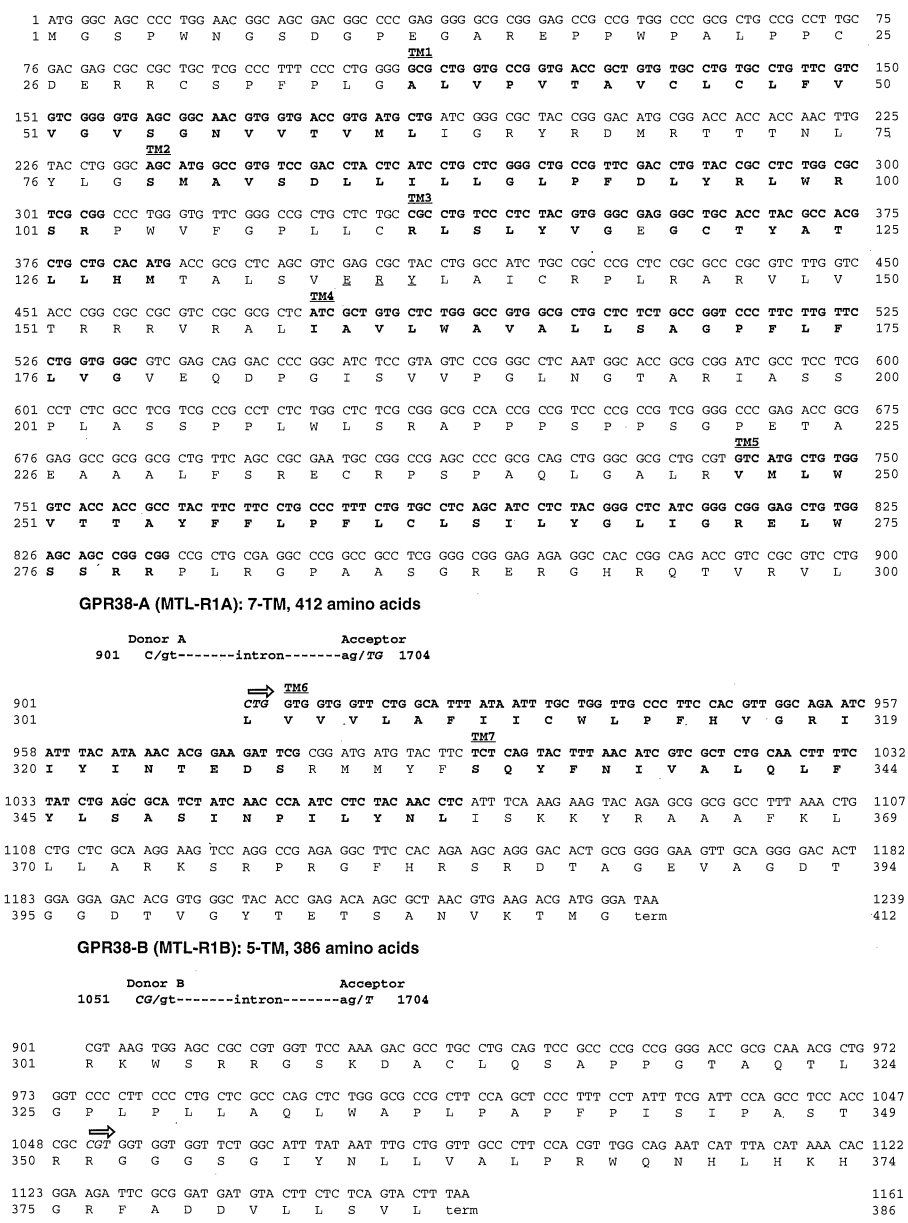

Fig. 1. Identification of two alternatively spliced forms of GPR38 (MTL-R1) (*39*). [A comparison of these two forms to human GHS-R and puffer fish clone 75E7 is available as supplementary material (*25*)]. GPR38-A (MTL-R1A) mRNA is formed by joining nucleotide 901 (donor A: imperfect splice donor C/gt) to nucleotide 1703 of the genomic GPR38 clone (perfect acceptor sequence-ag/TG). Formation of this splice junction results in the donor supplying C and the acceptor supplying TG to form the triplet codon for leucine- 301 (indicated by an arrow). GPR38-B mRNA is formed by joining of nucleotide 1051 (donor B: perfect splice donor-CG/gt) to the same acceptor as for GPR38-A. Formation of this splice junction results in the donor supplying CG and the acceptor supplying T to form the triplet codon for arginine-351 (indicated by an arrow). Donor and acceptor sites are diagrammed above each splice form with intron sequence shown in lower case type. Nucleotide and protein sequence numbering are given for the open reading frame sequence for GPR38-A and GPR38-B. TM represents predicted transmembrane domains; they are indicated by bold type and numbered 1 to 7.

Table 1. Pharmacology of native and cloned motilin receptors. Potencies are listed of various motilin peptides (analogs and truncated forms) and nonpeptide GI-active drugs against the native rabbit motilin receptor and the cloned human MTL-R1A. The rank order of potency for motilin-derived peptides correlates with respect to binding and functional assays performed on native rabbit motilin receptors (human ^{125}I-motilin binding and muscle strip contractility responses) and the cloned human receptor (human ^{125}I-motilin binding and aequorin bioluminescence). GI-active drugs whose mechanism of action is distinct from motilin (cisapride: serotonin receptor mixed agonist and antagonist; metoclopromide: dopamine receptor antagonist) do not bind or functionally activate the cloned human MTL-R1A at doses as high as 10 μM. Values for the human MTL-R1A are from a single experiment repeated three times ±10 SEM. Native rabbit motilin receptor data are compiled from (*1, 33, 34*). NA denotes data not available. Residues that differ from human motilin are underlined. Amino acid abbreviations are in (*39*) (L^n denotes norleucine).

Peptide or nonpeptide drug		Cloned motilin receptor (human)		Native motilin receptor (rabbit)	
		^{125}I-Motilin binding (IC_{50}, nM)	Aequorin assay (EC_{50}, nM)	^{125}I-Motilin binding (IC_{50}, nM)	Contractility assay (EC_{50}, nM)
	1 5 10 15 22				
Human 1–22	FVPIFTYGELQRMQEKERNKGQ	0.2	2.2	0.6	1
1–19	FVPIFTYGELQRMQEKERN	0.3	4.1	0.9	9
1–13	FVPIFTYGELQRM	0.3	3.8	15	100
1–12	FVPIFTYGELQR	0.9	72	76	324
1–11	FVPIFTYGELQ	142	138	150	642
1–9	FVPIFTYGE	>10,000	>10,000	3,900	>10,000
1–7	FVPIFTY	>10,000	<10,000	2,800	>10,000
1–5	FVPIF	>10,000	>10,000	3,800	>10,000
2–22	VPIFTYGELQRMQEKERNKGQ	33	626	NA	>1,000
10–22	LQRMQEKERNKGQ	>10,000	>10,000	417	9,000
^{13}Leu 1–22	FVPIFTYGELQRLQEKERNKGQ	0.9	3.6	0.7	7
^{13}nLeu^{14}Glu 1–22	FVPIFTYGELQRLEEKERNKGQ	0.3	10	NA	NA
^{3}Phe^{13}Leu 1–22	FVFIFTYGELQRLQEKERNKGQ	1.3	>10,000	NA	NA
Rabbit 1–22	FVPIFTYSELQRMQERERNRGH	0.2	13	NA	NA
Dog 1–26	FVPIFTHSELQKIREKERNKGQ	0.5	4	1	3
	IRNK				
Erythromycin		282	2,900	43	302
Roxithromycin		613	3,200	NA	NA
Metoclopromide		>10,000	>10,000	NA	NA
Cisapride		>10,000	>10,000	NA	NA

was greater than four times background (determined in the absence of GPR38 expression) was recorded for both the peptide motilin and the nonpeptide macrolide erythromycin, which was reported to be an agonist of native motilin receptors (*31*). Concentration dependence curves confirmed this observation (Fig. 2). The half-maximal effective concentration (EC_{50}) for human and porcine motilin was 2.2 nM, whereas erythromycin was less potent (EC_{50} of 900 nM), consistent with studies performed on native motilin receptors (*14*). Binding of ^{125}I-labeled human motilin (*32*) to cell membranes prepared from transfected cells that expressed GPR38-A (Fig. 3, A and B) showed that GPR38-A conferred high-affinity, saturable, and specific binding through two distinct binding sites (K_{d1} = 111 pM, K_{d2} = 960 pM; B_{max1} = 240 fmol/mg, B_{max2} = 520 fmol/mg). Overexpression of the cloned GPR38-A may deplete available G proteins needed for high-affinity binding, thus accounting for the observation of two binding sites (*33*). Both binding sites were G protein coupled (>80% inhibition of binding with 100 nM guanosine 5′-*O*-(3′-thiotriphosphate). On the basis of this data, GPR38-A was renamed motilin-R1A (MTL-R1A).

Structure-function analyses of a series of truncated motilin peptides (Table 1 and Fig. 3C) show that the motilin peptide minimally contains an NH_2-terminal region (amino acids 1 to 11) essential for biological activity, and a COOH-terminal α-helical domain that stabilizes the NH_2-terminus (*34, 35*). The rank order of potency of several motilin peptide analogs determined with the cloned MTL-R1A in the MTL-R1A functional and binding assays correlated with their reported potency measured by muscle-strip organ contraction and native receptor binding assays (Table 1). The NH_2-terminal fragments from amino acid 1 to 12 were similar in potency to full-length motilin (amino acids 1 to 22), and removal of the first amino acid resulted in a significant drop in potency. Erythromycin and the related macrolide roxithromycin gave median inhibitory concentration (IC_{50}) values, respectively, of 281 and 613 nM and EC_{50} values of 2900 and 3200 nM. The structurally unrelated GI motility–stimulating drugs metoclopromide and cisapride, which have affinity for dopamine and serotonin receptors, respectively, were inactive even at high (micromolar) doses.

MTL-R1A RNA was expressed in a subset of interstitial cells in the human duodenum, jejunum, and colon (Fig. 4) (*36*). These cells also demonstrated immunoreactivity for neuron-specific enolase. In the colon, most MTL-R1A–positive cells also expressed nitric oxide synthase (NOS), the enzyme responsible for production of nitric oxide, a major inhibitory gut neurotransmitter (Fig. 4C). In addition, a small number of cells stained positive for choline acetyl transferase,

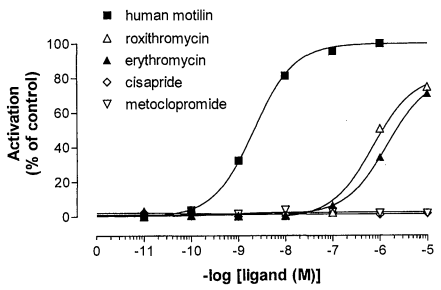

Fig. 2. Activation of the MTL-R1A receptor. Concentration-dependent increases in luminescence were observed with motilin, erthryomycin, and roxithromycin, but not with cisapride and metclopromide [EC_{50} (nM): 2.2, 2900, 3200, >10,000, >10,000, respectively]. A HEK-293 cell line stably expressing the bioluminescent indicator aequorin [HEK-293/aeq17; (*26*)] was transfected with a MTL-R1A expression plasmid (*28*). Cells were then exposed to the test ligands, and the integrated luminescence was recorded over 30 s. Activation (%) represents the luminescent response normalized to a maximum response evoked by 10 μM motilin. Data for each point on the dose-response curve represent the average of triplicate measurements for each sample (±10% SEM).

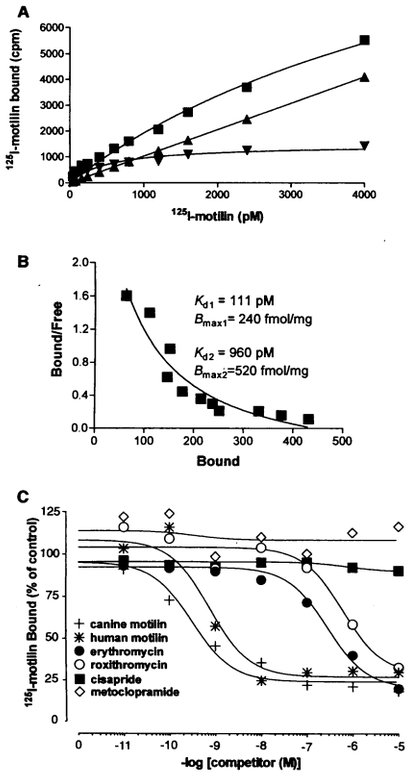

Fig. 3. Binding of human ^{125}I-motilin to crude membranes of HEK-293 cells expressing MTL-R1A. (**A**) Saturation isotherm. Increasing concentrations of radioligand were used to demonstrate saturable and specific binding: squares, total binding; triangles, nonspecific binding; and inverted triangles, specific binding. (**B**) Scatchard analysis of ^{125}I-motilin binding saturation isotherm suggests two binding sites of different affinities and binding capacities (bound units are femtomole per milligram of protein; free units are nanomolar). (**C**) Competition analysis for the binding of 0.2 nM ^{125}I-human motilin. Potencies obtained for inhibition of radioligand binding were in accord with results from the aequorin functional assay (Fig. 2) [IC_{50} (nM): 0.2 (human motilin), 0.5 (canine motilin), 282 (erythromycin), 613 (roxithromycin), >10,000 (cisapride), >10,000 (metclopromide]. Binding in the absence of excess unlabeled human motilin (100 nM) represents 100% of control binding. Binding data were analyzed by a nonlinear curve-fitting program (Prism, version 2.0; GraphPad Software, San Diego, CA). Results shown are the means (±SEM) of triplicate determinations.

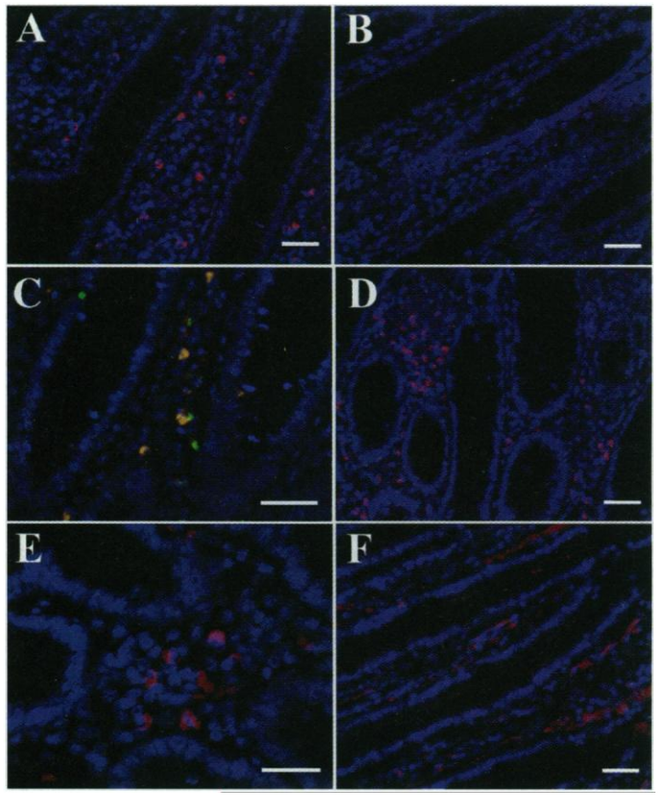

Fig. 4. Expression of MTL-R1A in the human GI tract by in situ hybridization and colocalization with nitric acid synthase–containing neurons: (**A**) colon, antisense probe for MTL-R1A; (**B**) colon, control sense probe for MTL-R1A; (**C**) colon, double-labeling to detect MTL-R1A (antisense probe) and NOS with antibody to NOS; (**D**) jejunum, antisense probe for MTL-R1A; and (**E** and **F**) duodenum, antisense probe for MTL-R1A. In situ signal is seen in red (Texas Red), NOS immunoreactivity in green (FITC), and all cell nuclei in blue (DAPI). All scale bars are 15 μm.

a synthetic enzyme for acetylcholine, the major stimulatory neurotransmitter of the gut. This expression pattern suggests that motilin may be stimulatory in the colon as well as in other levels of the human GI tract, because NOS-positive smooth muscle motor neurons are thought to inhibit distal circular smooth muscle activity in advance of a stimulatory bolus (*37*). In the duodenum, a separate population of cells having an elongate appearance consistent with smooth muscle were also MTL-R1A–positive (Fig. 4D). Finally, analysis of human MTL-R1A expression with mRNA extracted from additional regions of the GI tract revealed transcripts in esophagus, ileum, and jejunum (*38*).

To our knowledge, the receptor for a natural hormone has not been identified before by mass screening. The high amino acid sequence identity between MTL-R1A and GHS-R implies that motilin and a natural ligand for the GHS-R, which has yet to be identified, may also be related. The identification of the human MTL-R will aid the development of safe and selective motilin receptor agonists and antagonists useful for the treatment of GI disorders and help elucidate the role of motilin in human physiology.

References and Notes

1. C. Scarpignato, *Dig. Dis.* **15**, 112 (1997).
2. J. C. Brown, M. A. Cook, J. R. Dryburgh, *Can. J. Biochem.* **51**, 533 (1973).
3. Z. Itoh, *Peptides* **18**, 593 (1997).
4. P. Poitras, in *Gut Peptides: Biochemistry and Physiology*, J. H. Walsh and G. J. Dockray, Eds. (Raven, New York, 1994), pp. 261–304.
5. M. Tonini, *Pharmacol. Res.* **33**, 217 (1996).
6. Z. Itoh, M. Nakaya, T. Suzuki, *Am. J. Physiol.* **247**, G688 (1984).
7. F. H. Weber, R. D. Richards, R. W. McCallum, *Am. J. Gastroenterol.* **88**, 485 (1993).
8. Y. Seino *et al.*, *FEBS Lett.* **223**, 74 (1987).
9. C. T. Bond, G. Nilaver, B. Godfrey, E. A. Zimmerman, J. P. Adelman, *Mol. Endocrinol.* **2**, 175 (1988).
10. D. I. Daikh, J. O. Douglass, J. P. Adelman, *DNA* **8**, 615 (1989).
11. H. Yano *et al.*, *FEBS Lett.* **249**, 248 (1989).
12. T. Sakai *et al.*, *Regul. Pept.* **53**, 249 (1994).
13. I. Depoortere, G. Van Assche, T. L. Peeters, *Brain Res.* **777**, 103 (1997).
14. P. Poitras *et al.*, *Peptides* **17**, 701 (1996).
15. T. L. Peeters, V. Bormans, G. Vantrappen, *Regul. Pept.* **23**, 171 (1988).
16. I. Depoortere and T. L. Peeters, *ibid.* **55**, 227 (1995).
17. ———, G. Vantrappen, *Peptides* **14**, 1153 (1993).
18. I. DePoortere and T. L. Peeters, *Am. J. Physiol.* **272**, G994 (1997).
19. A. D. Howard *et al.*, *Science* **273**, 974 (1996).
20. K. K. McKee *et al.*, *Genomics* **46**, 426 (1997).
21. R. G. Smith *et al.*, *Endocr. Rev.* **18**, 621 (1997).
22. Inspection of the GPR38 genomic DNA sequence revealed two potential mRNA splice sites corresponding to consensus boundaries for exon-intron junctions [P. Senapathy, M. B. Shapiro, N. L. Harris, *Methods Enzymol.* **183**, 252 (1990)]. An imperfect donor site [TGC/gt; perfect donor sequence (CCG/gt)] was found at nucleotides 1929 to 1931 [of the 3068–base pair (bp) genomic fragment of GPR38 including 5′ untranslated region and intron]; a perfect splice-donor site was found at nucleotides 2080 to 2082; and a perfect splice-acceptor site [sequence (pyrimidine-rich stretch ag/TG)] was found at nucleotide 2729. To determine which splice forms exist naturally, we performed RACE on polyadenylated [poly(A)$^+$] mRNA isolated from human thyroid gland, and we conducted RT-PCR on HEK-293/aeq17 cells transfected with an expression plasmid containing the GPR38 genomic DNA intron. Directional RACE reactions were conducted on 1 μg of thyroid poly(A)$^+$ mRNA that contained RNA encoding GPR38 (*20*). Primers were synthesized corresponding to the 5′ end of the coding region of GPR38 (AP1: 5′-CCATCCTAATACGACTCACTATAGGGC) and the 3′ end of the GPR38 coding region including the translation termination codon TAA (5′ RACE1: 5′-TTATCCCATCGTCTTCACGTTAGCGCTTGTCTC). Conditions (Marathon cDNA, Touchdown PCR; Clontech, Palo Alto, CA) were as follows: 94°C for 1 min; 5 cycles of 94°C for 30 s and 72°C for 4 min; 5 cycles of 94°C for 30 s and 70°C for 4 min; and 25 cycles of 94°C for 20 s and 68°C for 4 min. An ~1.4-kb amplified product was identified that hybridized with a ^{32}P-labeled probe derived from the TM2 to TM4 region (3F/4R probe) of GPR38. This product was subcloned

into PCR-Script vector (Invitrogen) and sequenced. We confirmed these results using RT-PCR by transfecting an expression plasmid containing the GPR38 gene (the complete open reading frame interrupted by a single intron of ~0.7 kb) into HEK-293/aeq17 cells. RNA was then isolated [poly(A)$^+$ Pure Kit, Ambion] and subjected to Northern (RNA) blot analysis with the 3F/4R probe, which revealed two hybridizing bands: a 2.4-kb band containing the unspliced intron, and a ~1.4-kb band containing spliced forms. RT-PCR was then performed (Superscript 2 One-Step Kit, Life Technologies) on mRNA from transfected HEK-293/aeq17 cells with the forward primer 5′ RACE1 and reverse primer 3′ RACE2 (TM5 region: 5′-CTGCCCTTTCTGTGCCTCAGCATCCTCTAC). An ~500-bp product was cloned (TA vector pCR2.2, Invitrogen, Carlsbad, CA), sequenced, and found to be a mixture of both spliced forms. Assembly of the complete open reading frame for GPR38-A without intronic sequence was performed by ligation of an exon 1 fragment (Not I digestion of a MTL-R1A plasmid containing the intron in pCDNA-3) to pCDNA-3.1 containing a Not I–EcoR I exon 2 fragment.

23. W. C. Probst, L. A. Snyder, D. I. Schuster, J. Brosius, S. C. Sealfon, *DNA Cell Biol.* **11**, 1 (1992).
24. S. Brenner *et al.*, *Nature* **366**, 265 (1993).
25. GenBank database [D. Benson, M. S. Boguski, D. J. Lipman, J. Ostell, B. F. Ouellette *Nucleic Acids Res.* **26**, 1 (1998)] assession numbers are as follows: human GHS-R (HSU60179), human GPR38-A (MTL-R1A) (O43193), and fish clone 75E7 (AF082210). The alignment data are available at www.sciencemag.org/feature/data/1039909.shl
26. D. Button and M. Brownstein, *Cell Calcium* **14**, 663 (1993).
27. K. K. McKee *et al.*, *Mol. Endocrinol.* **11**, 415 (1997).
28. Measurement of GPR38-A activity in the aequorin-expressing stable reporter cell line HEK-293/aeq17 was made with a Luminoskan RT luminometer (Labsystems, Gaithersburg, MD). HEK-293/aeq17cells (8×10^5 cells plated 18 hours before transfection) were transfected with 11 μg of human GPR38-A plasmid DNA in the presence of 132 μg of lipofectamine (Life Technologies, Gaithersburg, MD). After 40 hours, the apo-aequorin in the cells was converted to holo-aequorin by incubation for an additional 1 hour in the presence of the essential chromophore coelenterazine (10 μM; Molecular Probes, Eugene, OR) under reducing conditions (300 μM reduced glutathione) in ECB buffer [140 mM NaCl, 20 mM KCl, 20 mM Hepes-NaOH, pH 7.4, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and bovine serum albumin (BSA; 0.1 mg/ml)], transferred into ECB buffer, washed once, and resuspended to 500,000 cells/ml. A 100-μl sample of cell suspension (corresponding to 5×10^4 cells) was then injected into the test ligand plate (96-well format), and the integrated light emission was recorded over 30 s, in 0.5-s units. Lysis buffer (20 μl) (0.1% final Triton X-100 concentration) was then injected and the integrated light emission recorded over 10 s, in 0.5-s units. The fractional response values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response.
29. To place an NH_2-terminal FLAG epitope in frame with the GPR38-A coding sequence, we constructed a mammalian expression vector by ligating a Pme I fragment excised from the pcDNA-1.1 expression vector (Invitrogen, Carlsbad, CA) containing GPR38 into the Eco RV site of pFLAG/CMV-2 vector (Kodak Imaging Systems, New Haven, CT). After lipofection (Life Technologies, Gaithersberg, MD) of this plasmid into HEK-293/aeq17 cells, a protein of the expected size (~45 kD) was detected in crude cell membranes by protein immunoblot analysis with the M1 monoclonal antibody to FLAG (Kodak Imaging Systems).
30. Ligand plates were assembled from commercial sources (Research Biochemicals International, Natick, MA; Phoenix Pharmaceuticals, Mountain View, CA) and contained an assortment of nonpeptide small molecules: for example, adenosines/purinerigics (theophylline, reserpine, phenyltoin, phenylbutazone), adrenergics/histaminergics (alprenolol, prazocin, phentolamine, histamine, cimetidine), cholinergics/ion channel modulators (carbachol, atropine, amiloride, diltiazem, Bay K-8644), dopaminergics (ergocristine, haloperidol, clozapine bromocryptine), glutamatergics (aspartic acid, capsaicin, glutamic acid, kainic acid, quisqualic acid), opioids (levallorphan, natrexone, norcodeine, nalaxone), serotonergics (ketanserin, quipazine, mianserin, tryptophan), enzyme inhibitors/GABAergics (captopril, papaverine, baclofen, allopurinol, indomethicin, erythromycin), and peptides (deltorphin, thrombin, cortistatin, amylin, neuromedin B, guanylin, neuropeptide EI, enterostatin, dynorphin A, amyloid b-protein, secretin, β-MSH, endothelin-3, GLP-1, GLP-2, TRH, adrenomedullin, BNP-32, ANF, katacalcin, galanin, pancreastatin, PACAP, γ-MSH, urotensin, calcitonin, PTH, CRF, prodynorphin, vasopressin, motilin, valosin, NPY 13-16, NPY 3-36, IL-8, thymosin, C5a, RANTES, GnRH, oxytocin, substance P, VIP, somatostatin, bombesin, b-endorphin, GHRP-6, endothelin-1, ACTH, angiotensin-2, bradykinin, fMLP, neurotensin, glucagon, CGRP, [des-Arg-9]bradykinin, endothelin-2, and gastrin).
31. T. Peeters *et al.*, *Am. J. Physiol.* **257**, G469 (1989).
32. Human motilin (COOH-terminally amidated; Research Genetics, Huntsville, AL) was radiolabeled with ^{125}I at Tyr^7 to a specific activity of ~2000 Ci/mmol (Woods Assay, Portland, OR). The binding of human ^{125}I-motilin to crude cell membranes prepared from HEK-293/aeq17 cells expressing MTL-R1A was determined as follows. Membranes were prepared on ice 48 hours after transfection. Cells were washed twice with 10 ml of phosphate-buffed saline and once with 1 ml of homogenization buffer (HB: 50 mM tris-HCl, pH 7.4, 10 mM $MgCl_2$). Cells were scraped into 10 ml of HB and then lysed with a Polytron device (Brinkmann, Syosset, NY; three bursts of 10 s at setting 4). The homogenate was centrifuged for 20 min at 11,000*g* at 0°C, then the crude membrane pellet was resuspended in HB supplemented with 1.5% BSA and kept on ice. Binding reactions were performed at 20°C for 1 hour in 0.5 ml containing 0.1 ml of membrane suspension (~1 μg of protein), 10 μl of human ^{125}I-motilin, 10 μl of competing peptide, and 380 to 390 μl of HB. Bound radioligand was separated by rapid vacuum filtration through GF/C grade filters (Whatman, Maidstone, UK) that were pretreated for 1 hour with 0.5% polyethylenimine. After application of the membrane suspension to the filter, the filter was washed three times with 3 ml of ice-cold HB buffer. Radioactivity bound to the filters was quantitated by gamma counting. Specific binding was defined as the difference between total binding and nonspecific binding that occurred in the presence of 100 nM unlabeled human motilin.
33. T. Kenakin, *Pharmacol. Rev.* **48**, 413 (1996).
34. M. J. Macielag *et al.*, *Peptides* **13**, 563 (1992).
35. P. Miller *et al.*, *ibid.* **16**, 11 (1995).
36. In situ hybridization was performed on 8-μm-thick, formalin-fixed, paraffin-embedded sections, with a digoxigenin-labeled complementary RNA probe corresponding to the complete open reading frame of MTL-R1A with tyramide–Texas Red detection, as described [K. Petrukhin *et al.*, *Nature Genet.* **19**, 214 (1998)]. NOS was detected with an antibody and visualized with a fluorescein isothiocyanate (FITC)–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were counterstained for nuclear detection with 4′,6-diamidoino-2 phenylindole (DAPI). Human tissues were obtained from the National Disease Research Interchange (Philadelphia, PA).
37. A. J. Porter, D. A. Wattchow, S. J. Brookes, M. Costa, *Gastroenterology* **113**, 1916 (1997).
38. RNA encoding MTL-R1A was detected by RNase Protection Assay (RPA) (MAXIscript and HybSpeed RPA kits; Ambion, Austin, TX). The anti-sense MTL-R1A probe corresponded to nucleotides 1234 to 1516 of the human MTL-1A with ~60 nucleotides of vector sequence (pLitmus 28, New England Biolabs, Beverly, MA). Poly(A)+ mRNA from tissues was purchased from Clontech (Palo Alto, CA).
39. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and L^n, n Leu.

9 March 1999; accepted 24 May 1999