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Molecular cloning of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells

(neuropeptide receptor/growth factor receptor/autocrine growth)

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ABSTRACT **The mammalian bombesin-like peptides gastrin-releasing peptide (GRP) and neuromedin B regulate numerous and varied cell physiologic processes in various cell types and have also been implicated as autocrine growth factors influencing the pathogenesis and progression of human small cell lung carcinomas. We report here the molecular characterization of the bombesin/GRP receptor. Structural analysis of cDNA clones isolated from Swiss 3T3 murine embryonal fibroblasts shows that the GRP receptor is a member of the guanine nucleotide binding protein-coupled receptor superfamily with seven predicted hydrophobic transmembrane domains. *In vitro* transcripts from cloned cDNA templates encompassing the predicted protein coding domain, when injected into *Xenopus* oocytes, resulted in expression of functional GRP receptors. The predicted amino acid sequence of the open reading frame in cDNA clones matches the amino-terminal sequence as well as the sequence of four tryptic fragments isolated from the purified protein. Expression of the GRP receptor cDNA in model systems potentially provides a powerful assay for the development of subtype-specific receptor antagonists that may prove to be of therapeutic importance in human small cell lung carcinoma.**

The mammalian bombesin-like peptides gastrin-releasing peptide (GRP) and neuromedin B (NMB) are regulatory peptides of importance in a wide variety of cell physiologic processes including secretion, smooth muscle contraction, and modulation of neuron firing rate (for review, see refs. 1 and 2). In addition, bombesin-like peptides can function as growth factors in Swiss 3T3 murine embryonal fibroblasts (3) and have been implicated as autocrine growth factors in the pathogenesis of some human small cell lung carcinomas (4). The bioactivities associated with the mammalian bombesin-like peptides are mediated by high-affinity binding to cell surface receptors present on many target cells (for review, see ref. 1). The properties of high-affinity bombesin/GRP receptors found in relatively high numbers (≈100,000 receptors per cell) on Swiss 3T3 fibroblasts have been extensively studied (5), with subsequent purification of the protein to near homogeneity (6). Molecular cloning of the gene encoding the Swiss 3T3 bombesin/GRP receptor is the next step to understanding the diversity and function of mammalian bombesin-like peptides and their receptors in physiologic and pathologic processes. In this paper, we report the isolation and characterization of cDNA clones§ encoding the Swiss 3T3 bombesin/GRP receptor, defining the structure of the receptor polypeptide. The clones obtained should provide a basis for further molecular analysis of the structure, function, and expression of receptors in Swiss 3T3 cells. In addition, these clones provide a means for a similar analysis of this receptor and other related bombesin peptide receptors expressed in the various cell types known to respond to this family of peptides.

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MATERIALS AND METHODS

Receptor Protein Purification and Peptide Sequencing. The GRP receptor was solubilized from Swiss 3T3 cell membranes in an active form and purified about 90,000-fold to near homogeneity using a combination of wheat germ agglutinin-agarose and ligand affinity chromatography as described in detail elsewhere (6). The purified receptor displayed a K_d for GRP (0.036 nM) that was about the same as that determined for the receptor in the Swiss 3T3 membranes. A partial amino acid sequence of the amino terminus of the purified receptor was determined by sequential Edman degradation. The sequence obtained was Ala-Pro-Asn-X_{aa4-7}-Leu-Asn-Leu-Asp-Val-Asp-Pro-Phe-Leu-Ser. To obtain additional sequences, 40 pmol of purified receptor (about 1.6 μg) was digested with 0.4 μg of trypsin for 22 hr at 37°C, and the peptides generated were fractionated by reverse-phase HPLC using a C_4 column (Brownlee Lab Aquapore Butyl) and a linear gradient of 0.05% trifluoroacetic acid in water to 0.05% trifluoroacetic acid in 100% (vol/vol) acetonitrile. Peaks in the effluent fractions were detected at 215 nm and subjected to automated sequence analysis on an ABI model 475A gas-phase sequencer, as described (7), equipped with an ABI model 120A on-line detection HPLC system for identification of phenylthiohydantoin amino acid derivatives. The sequences determined for four internal tryptic peptides were as follows: I1, Val-Pro-Asn-Leu-Phe-Ile-Ser-Ser-Leu-Ala-Leu-Gly-Asp-Leu-Leu; I2, Pro-Phe-Ile-Gln-Leu-Thr-Ser-Val-Gly-Val-Ser-Val-Phe; I3, Met-Ala-Ser-Phe-Leu-Val-Phe-Tyr-Val-Ile-Pro-Leu-Ala-Ile-Ile-Ser-Val; I4, Asn-Pro-Ser-Ala-Thr-Phe. Assignment of the amino acids in the first few cycles of degradation from the tryptic fragments was ambiguous due to the low levels of peptides available for sequencing (1–2 pmol). However, in all four cases, the cycle number of the amino acids determined for each tryptic fragment was consistent with the distance from a preceding basic amino acid predicted in the cDNA sequence.

Isolation of cDNA Clones. A cDNA library enriched for GRP receptor cDNA clones was constructed in λgt10 using methods described in detail (8). Briefly, 10 μg of hexamer-primed Swiss 3T3 cDNA was hybridized to 250 μg of BALB

Abbreviations: GRP, gastrin-releasing peptide; NMB, neuromedin B; G protein, guanine nucleotide binding protein.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M57922).

3T3 mRNA to a R_0t [initial concentration of RNA (mol of nucleotide per liter) × time (sec)] of about 3000. The unhybridized single-stranded Swiss 3T3 cDNA was purified by hydroxyapatite chromatography and hybridized to an additional 250 μg of BALB 3T3 mRNA to a R_0t of about 3000. cDNA that remained single stranded after the second round of differential hybridization was purified again by hydroxyapatite chromatography and was hybridized to 50 μg of Swiss 3T3 mRNA to generate RNA·DNA heteroduplexes. The heteroduplexes were purified by a third round of hydroxyapatite chromatography, and the RNA strand was converted to DNA using RNase H and DNA polymerase I. The duplex cDNA ends were "polished" with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase in a single reaction. An amplification linker with the following structure,

5'-AGCTAGAATTCGGTACCGTCGACC -3'

3'-TCTTAAGCCATGGCAGCTGGp-5',

was blunt-end ligated to the termini of the cDNA. Excess adaptors were removed by Sepharose 4B chromatography, and the linker-ligated cDNA was concentrated by ethanol precipitation. cDNA (10 ng) was enzymatically amplified using 30 cycles of polymerase chain reaction (94°C, 1 min; 37°C, 1 min; 72°C, 2 min) in a Perkin–Elmer/Cetus thermal cycler with 5 units of *Thermus aquaticus* polymerase and an amplification primer complementary to the short strand of the adaptor (5'-AGAATTCGGTACCGTCGACC-3'). After amplification, the product was size-fractionated and purified by gel electrophoresis followed by electroelution and cloned into λgt10 to construct the library.

An end-labeled nondegenerate 44-nucleotide-long antisense oligonucleotide whose sequence was based on codon usage frequency (9) of amino acids from the I3 peptide (Fig. 1) (5'-ATGATGGCCAGGGGGATCACATAGAAGACCAGGAAGGAGGCCAT-3') was used to screen 10^5 clones from the enriched library as described (11). Hybridizing clones were plaque-purified and subcloned into plasmid vectors as described (12) and analyzed by double-stranded DNA sequencing by using the Sequenase 2.0 kit (United States Biochemical) as recommended by the manufacturer. Two of the five clones analyzed had a long open reading frame that contained the amino acid sequence of the I3 peptide.

To obtain a single cDNA clone that encoded the uninterrupted open reading frame, a gene-specific oligonucleotide complementary to the predicted 3' untranslated region of GRP receptor mRNA was synthesized and used to prime first-strand cDNA synthesis. From this source of first-strand cDNA, a library of clones was generated in λgt10 as described (13). Ten clones were identified that extended into the 5' untranslated region beyond the sequences corresponding to the amino-terminal peptide and that, therefore, contained the entire open reading frame. Two of these clones were subcloned and sequenced.

Expression of the Cloned GRP Receptor in *Xenopus* Oocytes. Oocytes were injected with 20 ng of RNA transcribed (and capped) *in vitro* from a GRP receptor cDNA clone template (nucleotides −139 to 1322, Fig. 1) by using Sp6 RNA polymerase as recommended by the manufacturer (Promega). Oocytes were voltage-clamped at a holding potential of −60 mV, and the ligand-dependent Cl^- current was measured as described (14).

Northern Blot Analysis of mRNAs. Poly(A)$^+$ mRNA was prepared from either cell lines or tissues, resolved by electrophoresis on formaldehyde/agarose gels, blotted to nitrocellulose membranes, hybridized to a GRP receptor cDNA probe (nucleotides −139 to 1322, Fig. 1) that was labeled by nick-translation, washed, and subjected to autoradiography for 2 days as described (12). The RNA blot containing mouse mRNAs (see Fig. 3*B*) was washed at high stringency (65°C in 30 mM NaCl/3 mM sodium citrate/0.1% SDS, pH 7.0), and the RNA blot of rat mRNAs (Fig. 3*C*) was washed at 52°C in the same wash solution.

RESULTS AND DISCUSSION

A strategy designed to enrich for GRP receptor cDNA clones by subtractive cDNA library construction was used to account for the possibility that the GRP receptor mRNA might be a low-abundance transcript in cells expressing the gene. Ligand-binding studies (5) established that Swiss 3T3 murine embryonal fibroblasts display about 100,000 high-affinity GRP receptors per cell, whereas a similar murine fibroblast cell line (BALB 3T3) does not express the receptor at detectable levels. Furthermore, mRNA isolated from Swiss 3T3 cells elicited an electrophysiological response after injection and expression in *Xenopus* oocytes, whereas BALB 3T3 mRNA did not (data not shown). These observations indicate that the GRP receptor mRNA should be one of a limited number of transcripts present in Swiss 3T3 but absent from BALB 3T3. Thus, elimination of most cDNAs common to both cell lines would enrich for cDNAs encoding the GRP receptor.

The Swiss 3T3 GRP receptor protein was purified to near homogeneity (6), and amino acid sequences from the amino terminus and several internal tryptic fragments were determined (Fig. 1). A polymerase chain reaction-amplified subtracted cDNA library was constructed using Swiss 3T3 mRNA for the first-strand template followed by subtractive hybridization with an excess of BALB 3T3 mRNA. This enriched library was screened with an oligonucleotide probe designed to be specific for sequences encoding an internal tryptic fragment, Met-Ala-Ser-Phe-Leu-Val-Phe-Tyr-Val-Ile-Pro-Leu-Ala-Ile-Ile-Ser-Val, isolated from the purified GRP receptor (Fig. 1). Two positive clones in 100,000 subtracted library members were purified, and nucleotide sequence analysis confirmed that these clones had an open reading frame that contained the amino acid sequence of the I3 peptide. The cDNA clone inserts were used to rescreen the library, and 11 overlapping cDNA clones were isolated and sequenced defining the carboxyl-terminal end of a long open reading frame common to all of the clones. To obtain a clone containing the entire open reading frame, a gene-specific oligonucleotide complementary to the 3' untranslated region of the putative GRP receptor mRNA (as determined by sequencing previously isolated cDNA clones) was used to prime first-strand cDNA synthesis, and a library of clones enriched for GRP receptor cDNAs was generated as described (13). Ten clones were isolated that extended across the entire open reading frame into the 5' untranslated region, several of which were sequenced (Fig. 1).

Analysis of the nucleotide sequence revealed a single long open reading frame encoding a 384-amino acid protein with a predicted molecular mass of 43 kDa. The partial amino acid sequences of four tryptic fragments isolated from a digest of the purified GRP receptor protein were identified within the predicted protein sequence (Fig. 1), establishing that the cDNA clone corresponded to the protein purified by high-affinity binding to GRP. In addition, the amino-terminal sequence derived from the purified protein matched the predicted sequence from cDNA analysis at amino acids 2–4 (Ala-Pro-Asn) and 9–18 (Leu-Asn-Leu-Asp-Val-Asp-Pro-Phe-Leu-Ser), unambiguously defining the amino terminus of the Swiss 3T3 GRP receptor (Fig. 1). Seven potential sites of N-linked glycosylation (Asn-Xaa-Ser/Thr) were present in the open reading frame, consistent with reports that the Swiss 3T3 GRP receptor is highly glycosylated and that its apparent molecular mass on SDS gels is reduced from 75 kDa to about 45 kDa by N-Glycanase treatment (6, 15) (Fig. 1).

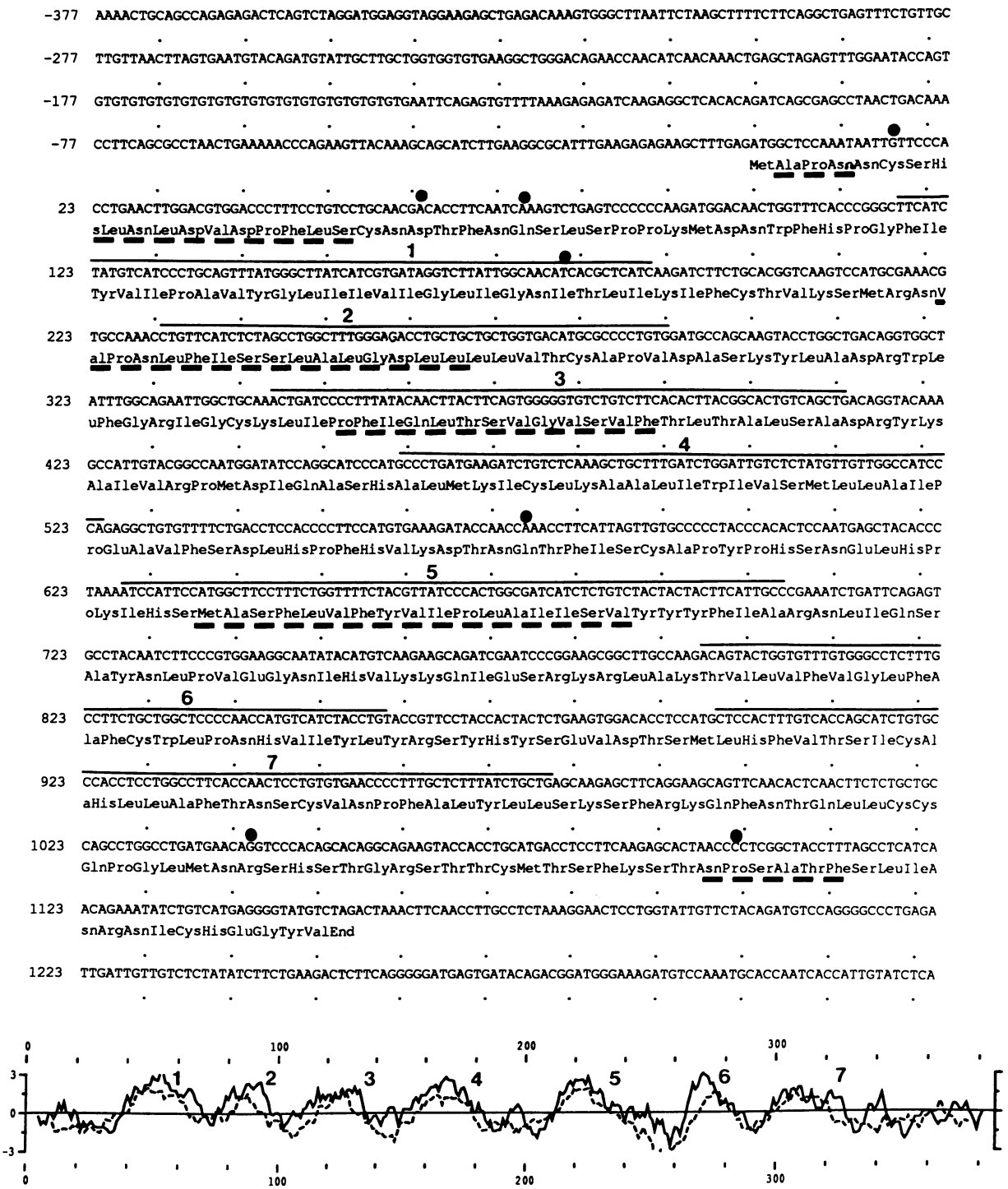

FIG. 1. (*Upper*) Nucleotide sequence and predicted amino acid sequence of the Swiss 3T3 GRP receptor cDNA clone. Heavy dashed underlines denote the amino-terminal and internal tryptic peptide sequences derived from the purified protein that are found in the open reading frame of the cDNA clone. Thin overlines indicate the locations of seven predicted transmembrane domains, based on homology to other G-protein-receptor superfamily members and the hydropathy plot (*Lower*). The open reading frame defines a protein whose predicted molecular mass is 43 kDa. Large solid dots designate the potential sites for N-linked glycosylation. (*Lower*) Hydropathy plot generated using the PepPlot program (window = 20 amino acids) in the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (10) and a VAX computer. Positive regions are relatively hydrophobic, and negative regions are hydrophilic. Predicted hydrophobic transmembrane domains are numbered sequentially. Solid line, Kyte–Doolittle criterion; dotted line, Goldman criterion.

Previous studies had provided evidence for the involvement of guanine nucleotide binding proteins (G proteins) in the transmembrane signaling that followed activation of the Swiss 3T3 GRP receptor (16), as well as the bombesin receptors found on GH_4C_1 pituitary and HIT pancreatic islet cell lines and rat brain membranes (17). A hydropathy plot of the amino acids in the predicted protein sequence revealed seven hydrophobic segments corresponding to the seven transmembrane domains typically found in members of the superfamily of receptor proteins coupled to G proteins (18) (Figs. 1 and 2). Protein sequence comparison between the GRP receptor and other members of the family indicated closer similarity with the bovine substance K receptor (19) than with other superfamily members examined (Fig. 2).

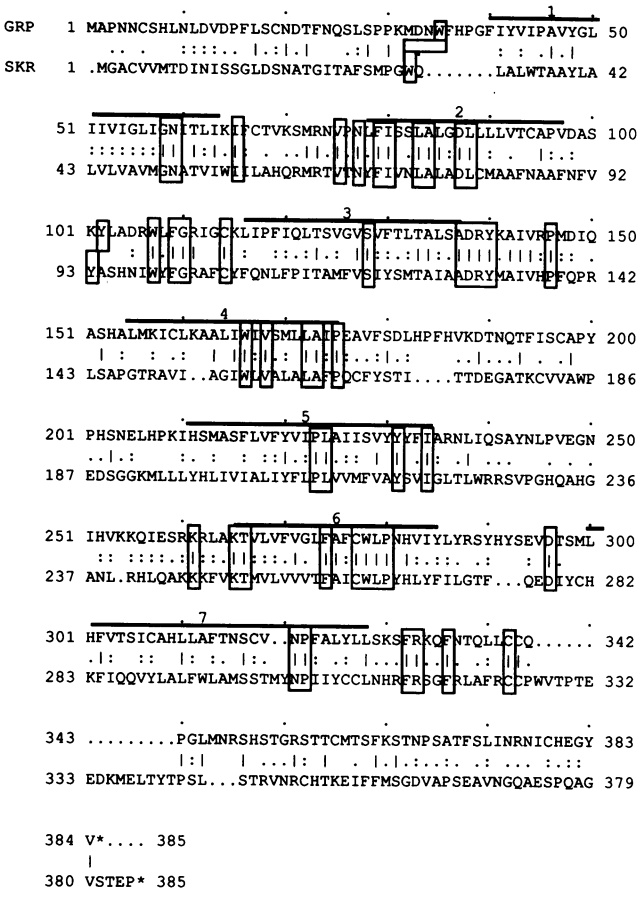

FIG. 2. Comparison of the structure of the GRP receptor protein with the bovine substance K receptor protein. The predicted amino acid sequence of Swiss 3T3 GRP receptor (Fig. 1) and bovine substance K receptor (19) are aligned to maximize homology using the GAP program Sequence Analysis Software Package of the Genetics Computer Group (10). Solid lines, amino acid identities; dotted lines, conservative substitutions at aligned positions as defined by the GAP program; vertical boxes, residues found in both GRP and substance K receptors that are also conserved in several other G-protein-coupled receptor sequences. [Other sequences used for comparison include bovine rhodopsin, human β-adrenergic receptor, and porcine cerebral and cardiac muscarinic receptors, which were aligned to highlight conserved residues (19).]

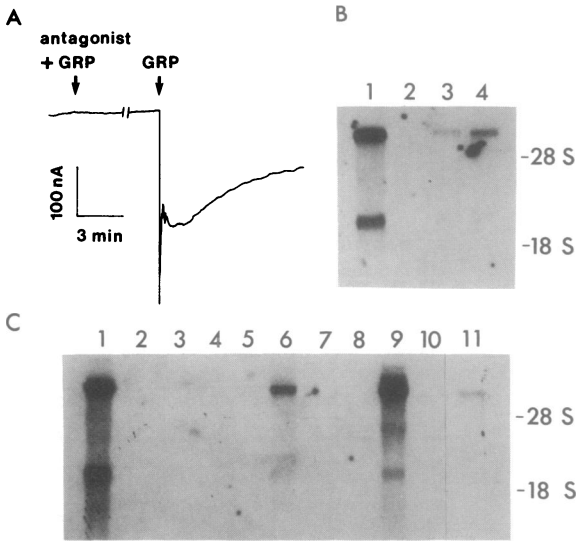

FIG. 3. Expression of the GRP receptor. (*A*) Electrophysiological response (chloride current versus time) of *Xenopus* oocytes to GRP application after expression of injected GRP receptor mRNA transcribed *in vitro* from a Swiss 3T3 cDNA template. The arrow indicates application of 1 μM GRP to an oocyte expressing the GRP receptor, with or without antagonist (10 μM). The average response in seven oocytes was 898 ± 135 nA. Oocytes responded to applications of GRP in concentrations as low as 1 nM. The response was also elicited by application of 1 μM NMB, although the magnitude of the response was typically severalfold lower than observed after application of 1 μM GRP. No response was noted when the antisense transcript was injected or when ligands unrelated to the bombesin-like peptides were applied. Addition of a specific GRP receptor antagonist {[D-Phe^6]bombesin-(6–13) ethyl ester} in a 10:1 molar ratio along with agonist abolishes the response. (*B*) RNA blot analysis of GRP receptor mRNA levels in mouse cell lines and adult male tissues. Two hybridizing species (7.2 kb and 3.1 kb) are identified by hybridization of the probe to 5 μg of poly(A)$^+$ mRNA isolated as follows. Lanes: 1, Swiss 3T3 mRNA; 2, BALB 3T3 mRNA; 3, mouse pancreas mRNA; 4, mouse brain mRNA. (*C*) RNA blot analysis of GRP receptor mRNA levels in rat cell lines and adult male rat tissues. Poly(A)$^+$ mRNA (2 μg) from each source was analyzed as described for *B*. Murine Swiss 3T3 and BALB 3T3 mRNA samples are also included for comparison. Lanes: 1, mouse Swiss 3T3; 2, mouse BALB 3T3; 3, rat stomach; 4, rat duodenum; 5, rat jejunum; 6, rat colon; 7, rat lung; 8, rat thymus; 9, AR42J rat pancreatic acinar cell line; 10, Rat-1 rat embryo fibroblast cell line; 11, rat brain mRNA.

Numerous amino acid residues conserved between the GRP receptor and the bovine substance K receptor are also shared with other G-protein-coupled receptor genes (boxed in Fig. 2), consistent with the assignment of the bombesin/GRP receptor as a member of this superfamily.

A cDNA clone containing the entire open reading frame of the GRP receptor (nucleotides −138 to 1322) was transcribed *in vitro* and the resulting transcript was injected into *Xenopus* oocytes for cell surface expression. A typical electrophysiologic response observed immediately after the application of GRP to an oocyte expressing the *in vitro* transcript is shown in Fig. 3*A*. Addition of a specific GRP receptor antagonist {[D-Phe^6]bombesin-(6–13) ethyl ester} (20) plus the agonist (10:1 molar ratio of antagonist/agonist) completely abolished the response. Oocytes injected with the antisense transcript exhibited no ligand-dependent response (data not shown). These studies further support the view that the protein sequence encoded by the cDNA clone constitutes a functional GRP receptor.

Expression of GRP receptor mRNAs was examined in a panel of samples isolated from various tissues and cell lines. RNA blot analysis shows two predominant transcripts [7.2 kilobases (kb) and 3.1 kb] in Swiss 3T3 cells detected under high-stringency wash conditions by the GRP receptor cDNA probe (Fig. 3 *B* and *C*). As expected, no hybridizing species were observed in BALB 3T3 mRNA samples. Detectable levels of GRP receptor mRNA were found in both mouse and rat brain mRNA, with somewhat less receptor mRNA observed in mouse pancreas mRNA (Fig. 3 *B* and *C*). In the rat gastrointestinal tract, GRP receptor mRNA was observed in the colon but not in stomach, duodenum, or jejunum. The rat pancreatic acinar cell line AR42J shows high levels of GRP receptor mRNA, but none was detectable in the Rat-1 embryo fibroblast cell line (Fig. 3*C*). These latter results are consistent with GRP-ligand-binding studies and oocyte expression studies performed in this laboratory indicating the presence of high levels of GRP receptors on AR42J but not Rat-1 cells (data not shown).

The RNA blot analyses reveal size heterogeneity in mRNA forms hybridizing to the GRP receptor probe. Two prominent mRNA species (7.2 and 3.1 kb) are observed in Swiss 3T3 mRNA. Both species are detected after a high-stringency wash (65°C, 0.1× SSC/0.1% SDS; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), indicating that both species are transcribed from a single gene. In one cDNA clone, a consensus polyadenylylation signal and poly(A) tract were

observed 3 kb downstream from the beginning of the 5′ untranslated sequences (data not shown). This clone precisely defines the structure of the shorter 3.1-kb mRNA form. No heterogeneity was noted in the structure or nucleotide sequence of the protein coding domain and 5′ untranslated region of six additional independent cDNA clones isolated from the Swiss 3T3 cDNA library. Thus these data suggest that the difference in length between the 7.2- and 3.1-kb mRNA forms is not found in either the 5′ untranslated or protein coding domain but probably represents different lengths of a rather long 3′ untranslated domain. It is interesting to note that the 7.2-kb mRNA form is much more prominent than the 3.1-kb form in RNA isolated from brain, pancreas, and colon, whereas the relative abundance of the two forms is nearly equal in the Swiss 3T3 cell line. In addition, the AR42J mRNA contains an additional hybridizing band (about 4.5 kb) not found in any other sample. Further experiments analyzing the structure of transcripts from various cells and tissues expressing the GRP receptor gene will be needed to precisely define the structure of the different forms of GRP receptor mRNA and to assess their relevance in determining the levels of GRP receptor protein found in cells expressing the gene.

These studies provide experimental approaches to understanding the role of the two mammalian bombesin-like peptides and their receptors in physiologic and pathologic processes. It is of interest to note that two bombesin receptor subtypes have been described in the rat esophagus and pancreas. The two subtypes show different binding affinities for GRP and NMB, as well as different relative potencies for the two ligands in bioassay systems (20, 21). The distribution of cells expressing GRP and NMB mRNAs is distinct in the brain (22), suggesting that the two structurally related mammalian bombesin-like peptides (GRP and NMB) have separate physiologic functions that may be mediated through different bombesin receptor subtypes present in the central nervous system. The Swiss 3T3 GRP receptor cDNA will allow unambiguous correlation between the distribution of the Swiss 3T3-type GRP receptor and these two peptides using *in situ* hybridization. This correlation will provide evidence to clarify the potential role of other bombesin receptor subtypes in the central nervous system, as well as defining their anatomical localization. Further, the Swiss 3T3 GRP receptor may have sequences similar to other less well characterized subtypes, allowing the isolation of cDNAs from genes encoding other mammalian bombesin receptors by low-stringency cloning techniques.

Previous studies have established the importance of GRP as a growth factor in Swiss 3T3 fibroblasts (3) and in maintaining the growth of some human small cell lung carcinomas (4, 23–25). The growth-promoting effect is mediated through ligand binding to the GRP receptor and can be diminished by addition of a monoclonal antibody that binds the carboxyl-terminal domain of the GRP peptide (4) or by GRP receptor antagonists (24, 25). The Swiss 3T3 GRP receptor cDNA clone described herein should allow straightforward isolation of human GRP receptor clones, defining the properties of the GRP receptor that potentially mediates autocrine growth in some lung carcinomas. Comparison of GRP receptor cDNAs from both malignant and normal sources will determine whether or not alterations in GRP receptor structure, function, or regulation are important molecular events in small cell lung carcinoma tumorigenesis or progression. Finally, cDNA clones for both the Swiss 3T3 and small cell lung carcinoma GRP receptors will allow the production of large quantities of this relatively rare protein in a purified form, greatly facilitating the identification and characterization of specific GRP receptor antagonists that may prove useful as future therapeutic agents in the treatment of small cell lung cancer.

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