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Bombesin Receptor Structure and Expression in Human Lung Carcinoma Cell Lines

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Abstract Mammalian bombesin-like peptides gastrin-releasing peptide (GRP) and neuromedin B (NMB) are regulatory neuropeptides involved in numerous physiologic processes, and have been implicated as autocrine and/or paracrine growth factors in human lung carcinoma. Three structurally and pharmacologically distinct bombesin receptor subtypes have been isolated and characterized: the gastrin releasing peptide receptor (GRP-R), the neuromedin B receptor (NMB-R), and bombesin receptor subtype-3 (BRS-3). The three receptors are structurally related, sharing about 50% amino acid identity. They are members of the G-protein coupled receptor superfamily with a seven predicted transmembrane segment topology characteristic of receptors in this family. The signal transduction pathway for GRP-R and NMB-R involves coupling to a pertussis-toxin insensitive G-protein, activation of phospholipase C (PLC), generation of inositol trisphosphate (IP3), release of intracellular calcium, and activation of protein kinase C. While all three bombesin receptors are activated by bombesin agonists, GRP-R, NMB-R, and BRS-3 have very different affinities for the mammalian bombesin-like peptides GRP and NMB, as well as bombesin receptor antagonists. The three bombesin receptor subtypes are expressed in an overlapping subset of human lung carcinoma cell lines. Any therapeutic strategy based on modulation of bombesin growth responses in human lung carcinoma would be well served to take into account the pharmacologic heterogeneity of the relevant receptors.

Key words: bombesin receptor, gastrin releasing peptide receptor, neuromedin B receptor, bombesin, autocrine growth

Bombesin is a tetradecapeptide originally purified from the skin of the European frog *Bombina bombina* [1]. Many bombesin-like peptides with related C-terminal octapeptide structures were subsequently isolated from various amphibians and classified into three subfamilies (bombesin, ranatensin, and phyllolitorin) based on the sequence of the last three residues in their amidated C-terminal domain [2]. Two mammalian bombesin-like peptides been identified and characterized: gastrin-releasing peptide (GRP) [3] in the bombesin subfamily, and neuromedin B (NMB) [4] in the ranatensin subfamily. As yet, no mammalian counterpart to the amphibian phyllolitorin peptides has been identified at either the gene or peptide level.

Mammalian bombesin-like peptides have been associated with a wide spectrum of physiologic effects, including regulation of secretion, growth, and neuromodulation [reviewed in ref. 5]. In normal tissues, bombesin-related peptides stimulate growth of normal bronchial epithelial cells [6] and endometrial stromal cells [7]. The growth stimulatory properties of bombesin in Swiss 3T3 fibroblasts [8] have been used to great advantage as a model system, providing significant insights into the biochemical and genetic changes leading to cell proliferation. Furthermore, bombesin can regulate reorganization of the actin cytoskeleton, an important component of many cellular processes including cell division, by stimulating accumulation of the small GTP-binding proteins Rho and Rac in the active GTP-bound state. Accumulation of activated Rac leads to membrane ruffling, while activated Rho results in stress fiber formation [9].

Several observations indicate that growth stimulation by mammalian bombesin-like peptides plays a significant role in human neoplasia. Immunocytochemical studies demonstrated the presence of bombesin-like immunoreactivity [10,11] and gastrin-releasing peptide mRNA [12]

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in many human small cell lung cancer (SCLC) tumors and cell lines. The clonal growth of some SCLC cell lines in soft agar was increased by addition of bombesin and gastrin-releasing peptide [13]. A neutralizing monoclonal antibody (2A11) directed against the C-terminal domain of bombesin, the peptide domain critical for receptor binding, attenuated growth of some human SCLC cells both as soft agar clones in vitro and nude mouse xenografts in vivo [14]. Potent bombesin receptor antagonists inhibited the growth of bombesin-receptor positive, but not receptor negative, SCLC cells in vivo and in vitro [15–17]. These findings indicate that mammalian bombesin-like peptides can function as autocrine growth factors of potential importance in the pathogenesis and progression of some human lung carcinomas.

Bombesin-mediated growth may also be relevant in other malignancies besides lung cancer. Immunohistochemical analysis of human prostate tumors revealed that about half of the specimens showed features of neuroendocrine differentiation, many of which were positive for bombesin-like immunoreactivity [18]. In a study of the human prostate cancer cell line PC-3, GRP receptors and bombesin-dependent growth stimulation were observed [19]. A bombesin receptor antagonist inhibited growth of nude mouse xenografts established using the prostate cancer cell line PC-82 [20].

Bombesin was also shown to stimulate the proliferation of human breast carcinoma cells in culture [21,22], or as nude mouse xenografts [23]. Expression of the gastrin releasing peptide gene [24] and GRP receptors [25] were reported in human breast cancer cell lines. The bombesin receptor antagonist RC-3095 was also shown to inhibit the growth of human breast cancer cell lines [26], suggesting the possibility that gastrin-releasing peptide may function as an autocrine or paracrine growth factor in some breast cancer cells. Given these findings, attenuation of bombesin-mediated responses, or intracellular pathways which mediate these growth-regulatory responses, may provide a means for interfering with the growth of bombesin-dependent cells in several different tumor types.

It has proven difficult to analyze the functional properties of bombesin receptors mediating these effects in the context of a human tumor cell. Typically, the number of receptors per cell is low, and often there is more than one receptor subtype expressed by the same cell. Both of these variables complicate functional and pharmacologic studies of the bombesin signalling pathway. To precisely determine the functional and pharmacologic properties of a specific receptor, it is very useful to design model systems where the receptor is expressed at uniformly high levels in the absence of other receptor subtypes with overlapping functional properties that could complicate the study. These model systems are developed by obtaining cDNA clones for the family of mammalian bombesin receptors, and expressing the receptors in appropriate host cells that do not express their own endogenous bombesin receptors.

Recently, we [27–30] and others [31,32] have cloned and characterized three structurally and pharmacologically distinct human bombesin peptide receptor subtypes: GRP-R, NMB-R, and bombesin receptor subtype-3 (BRS-3). In this study, the properties of these three bombesin receptor subtypes are summarized, and the pattern of expression in a panel of human lung carcinoma cell lines was studied using a very sensitive and specific assay involving reverse transcription of mRNA followed by polymerase chain reaction (RT/PCR) [33].

MATERIALS AND METHODS

Isolation and Characterization of Bombesin Receptor cDNAs

Procedures used for isolation and characterization of cDNA and genomic clones encoding the murine GRP-R [27,30], rat NMB-R [28], human GRP-R and NMB-R [29], and human BRS-3 [30] are found in the indicated references.

Reverse Transcription/Polymerase Chain Reaction (RT/PCR) Assay to Detect Bombesin Receptor mRNAs Expressed in Human Lung Cancer Cell Lines

Lung cancer cell lines were kindly provided by Drs. J. Minna and A. Gazdar, and their histological typing summarized in Corjay et al. [29]. Total RNA was isolated from cultured cells as described [34]. Total RNA was reverse transcribed using gene-specific antisense oligonucleotide primers complementary to 3′ untranslated sequences in receptor mRNAs (huGRP-R: 5′TTCCTGTCTAGCCATAAAGC 3′; huNMB-R: 5′GTTCTCTCCAGGTAGTGAGTT 3′; huBRS-3: 5′CCTGCACACAGCTTAGAGTC 3′). One microgram total RNA, 100 ng gene specific primer, and water were combined in 10 μl volume, incu-

bated at 70°C for 2 min, and placed on ice. The following reagents were added to the primer and RNA: 4 μl 5 × RT buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; Bethesda Research Labs, Gaithersburg, MD), 2 μl 0.1 M DTT, 4 μl 2.5 mM dGTP, dATP, dTTP, dCTP, 1 μl RNasin (Promega, Madison, WI), 1 μl Moloney-MLV™ reverse transcriptase (200 U/μl; Bethesda Research Labs). The reverse transcription reaction was incubated at 42°C for 1 h, and the enzyme heat inactivated by incubation at 70°C for 5 min. This cDNA served as template for the polymerase chain reaction (PCR).

In the PCR reaction, the gene-specific PCR primers for each receptor mRNA were resuspended at 100 ng/μl in water: GRP-R sense primer, 5'TTAAAGAAGGCAAAGAGC 3'; GRP-R antisense primer, 5'ATCTTCATCAGGGCATGGGA 3'; NMB-R sense primer, 5'GTGGGCGTTCAGTCCTCAGG 3'; NMB-R antisense primer, 5'ACTTCTGAAAACACCGCTTC 3'; BRS-3 sense primer, 5'GGCTCAAAGGCAGCCTCACT 3'; BRS-3 antisense primer, 5'AGTCTTCAGGATGGCATTGG 3'. The PCR sense and antisense primer pairs were chosen such that they spanned an intron, so that only a cDNA template, and not genomic DNA, could be successfully amplified to give a PCR product of the predicted size.

The PCR reaction was performed essentially as described, using a commercially available kit (GeneAmp™, US Biochemicals, Cleveland, OH). The reactions were assembled as follows: 1 μl cDNA from the reverse transcription reaction, 10 × PCR buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% [w/v] gelatin [Type A porcine]), 8 μl 2.5 mM dGTP, dATP, dTTP, dCTP, 1 μl each of the gene specific sense and antisense primers, 78 μl water, 1 μl Amplitaq™ Taq DNA polymerase (US Biochemicals), overlayed with 50 μl mineral oil. The reaction was cycled using a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT) for 35 rounds of: 94°C, 1 min; 50°C, 1 min, 72°C, 1 min. The reaction was extended for 5 min at 72°C at the end of the last cycle.

Ten microliter PCR product from each sample was analyzed by agarose gel electrophoresis using established methods [34]. The predicted sizes of the PCR products were: GRP-R, 586 bp; NMB-R, 617 bp; BRS-3, 489 bp. Agarose gels were blotted to nitrocellulose membranes using established methodology [34], and were baked at 80°C for 30 min to fix the transferred DNA. Membranes were hybridized to gene-specific oligonucleotide probes complementary to cDNA sequences located between the PCR primers (GRP-R, 5'-CCTGGCTGACAGATGGCTATTT-3'; NMB-R, 5'-CCCAGCAAGCCCACGGTGAT-3'; BRS-3, 5'-TCCTTCTGCAAGGTAGTGAG-3'). The primers were end-labelled using gamma-^{32}P-ATP (3,000 Ci/mMol) and T4 polynucleotide kinase as described [34]. Membranes were hybridized in 40% (v/v) formamide, 0.6 M NaCl, 60 mM NaCitrate, 20 mM Tris pH 7.4, 20 μg/ml denatured herring sperm DNA, 10% (w/v) dextran sulfate, 0.02% (w/v) bovine serum albumin, 0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinylpyrrolidone, 5 × 10^5 cpm/ml labelled oligonucleotide probe at room temperature (22°C) overnight. After hybridization, the filters were washed twice for 10 min each in several hundred ml of 0.3 M NaCl, 30 mM NaCitrate, pH 7.0, 0.1% (w/v) SDS, and twice in several hundred ml of 15 mM NaCl, 1.5 mM NaCitrate, pH 7.0, 0.1% (w/v) SDS for 10 min each at 32°C. After washing, the filters were exposed to Kodak X-AR film for several hours to generate autoradiograms.

RESULTS

Figure 1 shows an amino acid comparison of the amphibian peptides bombesin and ranatensin with their mammalian counterparts gastrin releasing peptide and neuromedin B, respectively. Gastrin-releasing peptide is most similar to bombesin, while neuromedin B is more similar to ranatensin. Note the structural similarity of all four peptides at their amidated C-termini (Trp-Ala-Val/Thr-Gly-His-Leu/Phe-Met-NH_2), and the lack of conserved sequence over the amino terminal domain of the peptides. The conserved C-terminus appears to be the critical domain for binding to high affinity receptors [35], conferring biological activity in mammalian systems; the remarkable conservation of this domain explains why the amphibian bombesin-like peptides elicit potent responses in mammals.

The nucleotide sequence of the coding regions of five mammalian bombesin receptor cDNAs is shown in Figure 2, with the sequences aligned to maximize homology. The corresponding predicted coding sequences of the same five receptor cDNAs are aligned in Figure 3. As expected, mammalian homologs of the same receptor (mouse GRP-R and human GRP-R, 90% identity; rat NMB-R and human GRP-R; 89% identity) are more similar in amino acid sequence

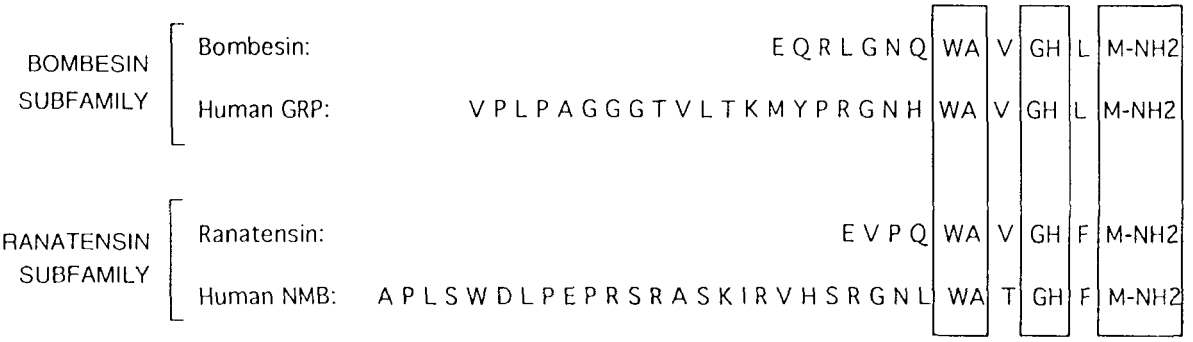

Fig. 1. Amino acid comparison of the amphibian peptides bombesin and ranatensin with their mammalian relatives GRP and NMB, respectively. Note the remarkable sequence identity over the seven C-terminal amino acids (boxed), the peptide region critical for receptor binding.

than two distinct receptor subtypes from the same species (human GRP-R and human NMB-R; 55% identity) (Fig. 3). The five receptors show the greatest degree of similarity in the seven hydrophobic transmembrane domains (TM I-VII), while the predicted extracellular domains (e1–e4) are less well conserved. These observations are consistent with the hypothesis that conserved residues in these domains may play an important role in determining the ligand binding pocket. Three conserved sites for potential phosphorylation by protein kinase C are also noted in intracellular domains 3 and 4 (vertical boxes with asterisks, Fig. 3).

The signal transduction pathway utilized by GRP-R (Benya et al., manuscript in preparation) and NMB-R expressed after transfection into Balb 3T3 fibroblasts [36,37] has been studied in detail. For both receptors, ligand binding results in activation of a pertussis toxin insensitive guanine nucleotide binding protein (G-protein), which activates phospholipase C resulting in an increase in inositol trisphosphate, followed rapidly by transient elevation in intracellular calcium. Due to the lack of a high affinity agonist, detailed characterization of the signal transduction properties of BRS-3 has not been performed. The bombesin-dependent BRS-3 electrophysiologic assay performed in *Xenopus* oocytes [30] monitors the opening of a calcium dependent chloride channel, suggesting that the BRS-3 signal transduction pathway also involves a transient elevation in intracellular calcium.

The binding properties of GRP-R and NMB-R transfected into Balb 3T3 fibroblasts are compared in Table I. Quantitative ligand displacement of [^{125}I]Tyr^{4}bombesin shows the distinct binding properties of these two receptors, with the rank order of affinity for binding to GRP-R (GRP, [D-Phe^6]bombesin (6–13) ethyl ester antagonist > NMB) differing from NMB-R (NMB > GRP, [D-Phe^6]bombesin (6–13) ethyl ester antagonist). Bombesin binds both receptors with high affinity (K_i = 1–3 nM). Neither GRP nor NMB appear to be high affinity ligands for BRS-3 [30,32]. Micromolar levels of GRP, NMB, or BN are needed to elicit responses from BRS-3 expressed in *Xenopus* oocytes [30], while nanomolar concentrations of high affinity ligands for GRP-R and NMB-R elicit responses in the same assay [27–29].

The pattern of expression of these three pharmacologically distinct bombesin receptors was examined in a panel of human small cell lung carcinoma cell lines. The expression of transcripts encoding the three human bombesin receptor subtypes (huGRP-R, huNMB-R, huBRS-3) was assayed by reverse transcription followed by polymerase chain reaction using gene-specific primers (RT/PCR). This assay is the most sensitive assay available for mRNA detection [33], and was chosen because the levels of GRP-R and NMB-R mRNAs detected in bombesin responsive cell lines were shown to be very low using a less sensitive but quantitative RNase protection assay in an earlier study [29]. Representative results are shown in Figure 4, and results from a panel of cell lines are summarized in Table II. Expression of GRP-R, NMB-R, and BRS-3 mRNA is widespread in human lung carcinoma cell lines, with most cell lines expressing at least one of the three receptors and many cell lines expressing more than one receptor (H345, for example).

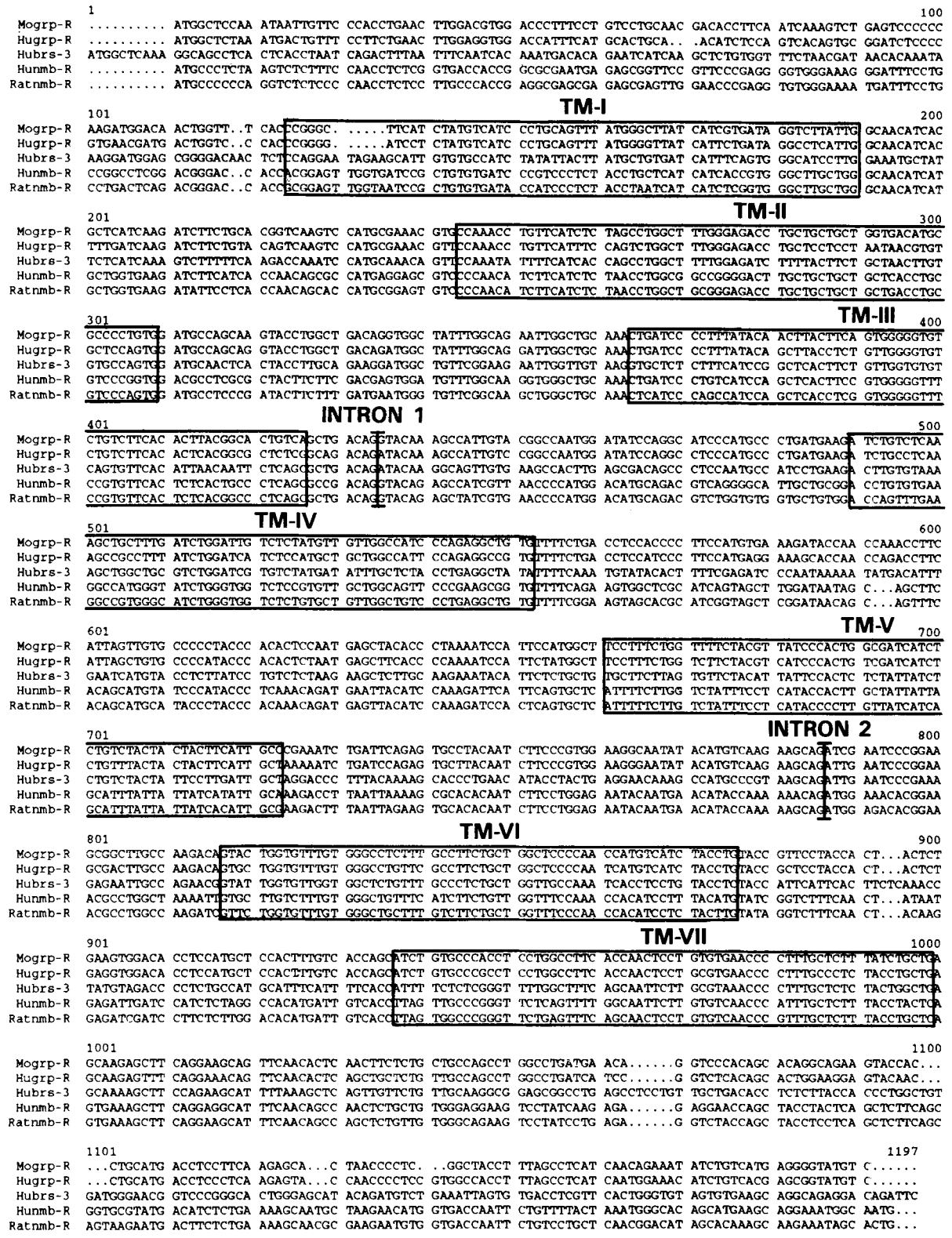

Fig. 2. Nucleotide sequence comparison of coding domains of five cloned mammalian bombesin receptors. The nucleotide sequences are aligned to maximize sequence identity at a givel position. Boxes designate sequences encoding the seven transmembrane protein domains characteristic of G protein-coupled receptors. The conserved location of two introns dividing the coding regions is indicated. The receptor sequences shown are as follows: Mogrp-R, mouse gastrin releasing peptide receptor [27]; Hugrp-R, human gastrin releasing peptide receptor [29]; Hubrs-3, human bombesin receptor subtype 3 [30]; Hunmb-R, human neuromedin B receptor [29]; Ratnmb-R, rat neuromedin B receptor [28].

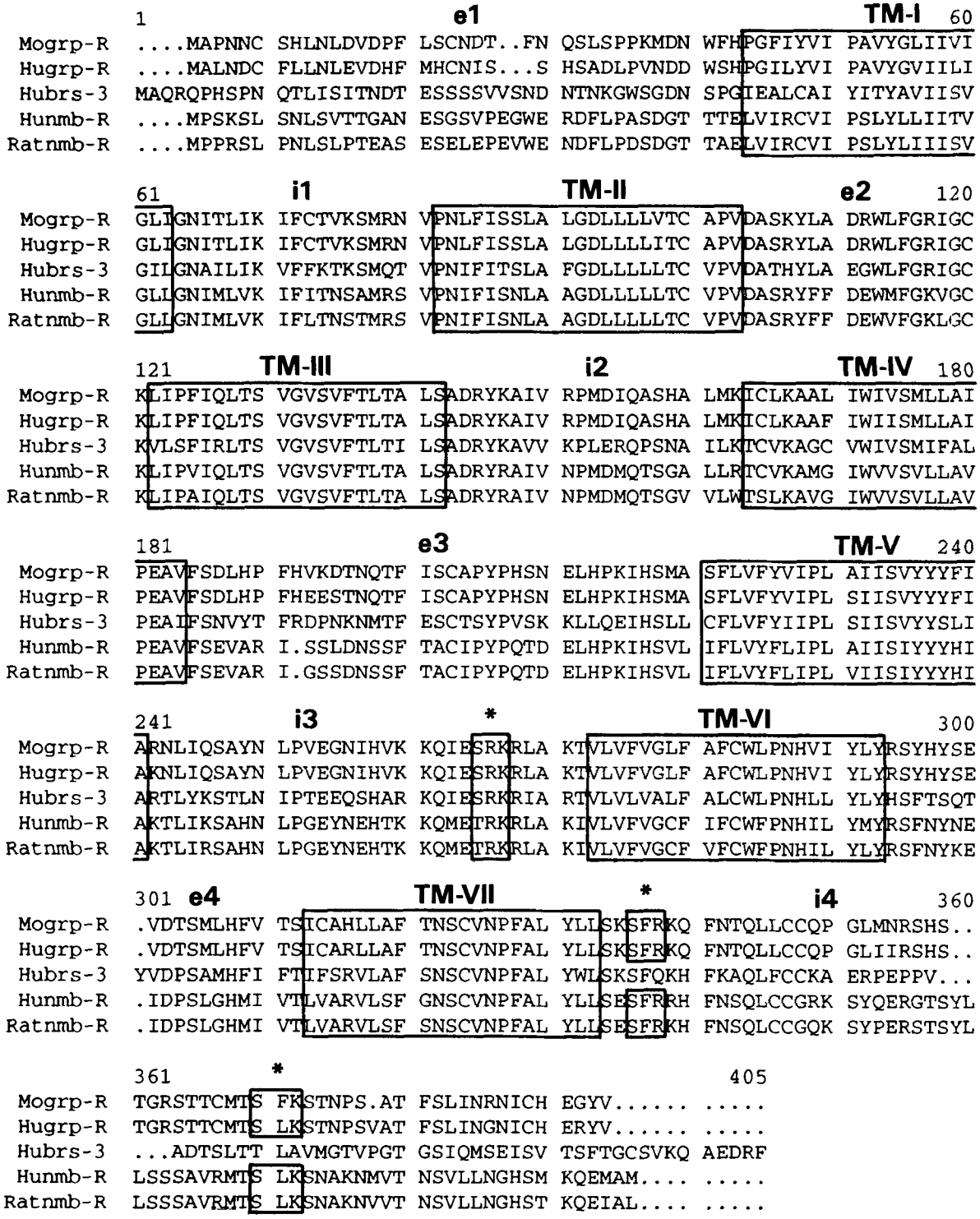

Fig. 3. Amino acid sequence comparison of five cloned bombesin receptors. The amino acid sequences are aligned to maximize sequence similarity. Note the remarkable sequence similarity in regions of the seven putative transmembrane domains (TM-I to TM-VII). Four extracellular domains (e1–e4) and four intracellular domains (i1–i4) are indicated, as well as three consensus protein kinase C phosphorylation sites (boxed, with asterisks) in the i3 and i4 domains which may mediate receptor desensitization by interfering with coupling to G-proteins.

TABLE I. Displacement of [^{125}I]Tyr^4 Bombesin Binding by GRP, NMB, and the Antagonist [D-Phe^6]Bombesin$_{6-13}$ Ethyl Ester*

Subtype	K_i (nM)		
	GRP	NMB	Antagonist
MoGRP-R	2	62	1.6
RatNMB-R	43	2	>1,000

Displacement of [^{125}I]Tyr^{4}bombesin binding by GRP, NMB, and the antagonist [D-Phe^6]bombesin$_{6-13}$ ethyl ester. The whole cell binding assay was performed as described [28]. Note the remarkable difference in affinity for the antagonist seen when GRP-R and NMB-R are compared. BRS-3 does not bind the labelled bombesin tracer with sufficient affinity to accurately measure displacement above background in this assay.

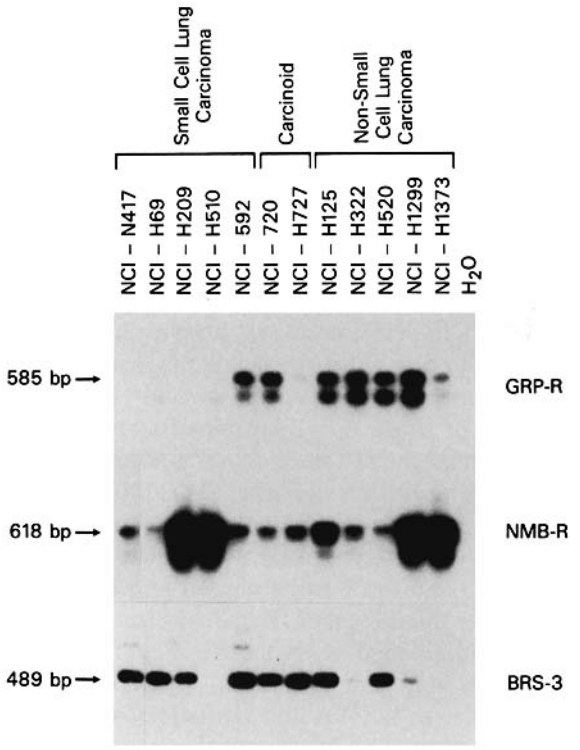

Fig. 4. RT/PCR analysis of bombesin receptor expression in a panel of human lung carcinoma cell lines. Total RNA samples isolated from cell lines was reverse transcribed, and the resulting cDNA subjected to polymerase chain reaction amplification using gene-specific primers for GRP-R, NMB-R, or BRS-3. The PCR product was resolved by agarose gel electrophoresis, blotted, and hybridized to a gene-specific oligonucleotide probe to detect receptor mRNA expression in the individual cell lines. Representative data is shown in this figure, and a summary of the data from all cell lines examined can be found in Table II. Note that most cell lines express at least one of the three bombesin receptors. Details of the method are provided in Materials and Methods.

TABLE II. Bombesin Receptor Subtype mRNA Expression in Lung Cancer Cell Lines as Determined by RT/PCR*

Cell line and morphological type	GRP-R	NMB-R	BRS-3
Small cell lung carcinoma			
NCI-H69	Tr	Tr	+
NCI-H82	–	+	–
NCI-H209	–	+	+
NCI-H345	+	+	–
NCI-N417	–	+	+
NCI-H510	Tr	+	–
NCI-N592	+	+	+
Carcinoid			
NCI-H720	+	+	+
NCI-H727	–	+	+
Non-small cell lung carcinoma			
NCI-H23	–	Tr	–
NCI-H125	+	+	+
NCI-H157	–	+	–
NCI-H226	Tr	+	–
NCI-H322	+	+	Tr
NCI-H358	Tr	+	+
NCI-H441	–	Tr	–
NCI-H460	–	Tr	–
NCI-H520	+	+	+
NCI-H661	–	+	–
NCI-H810	–	Tr	–
NCI-H1299	+	+	Tr
NCI-H1373	Tr	+	–

*(+), (Tr), and (–) indicate high, trace, and non-detectable levels of mRNA expression respectively. Bombesin receptor mRNA expression detected by RT/PCR in human lung cancer cell lines. The presence or absence of a detectable PCR product is indicated by a +; barely detectable expression is designated trace (tr); no expression is indicated by a –. Both small cell and non-small cell lung cancer cell lines were examined. The assay was performed as indicated in Materials and Methods.

DISCUSSION

Human lung tumor cell lines have proven to be a reliable source for the identification, isolation, and structural characterization of human bombesin receptor subtype cDNAs, where, in some examples, they are reported to play an important role in the pathogenesis or progression of neoplasia. Curiously, bombesin receptor mRNA levels are invariably low in all the human lung cancer cell lines studied. The explanation for the low expression is not clearly understood at present, but may be an important feature for exhibiting an autocrine or paracrine growth response. In quiescent Balb 3T3 cells expressing

high levels of transfected NMB-R (8×10^5 receptors/cell), the growth response is biphasic with apparent growth stimulation at low levels of peptide (less than 10 nM) and growth inhibition at higher levels (10–100 nM) [37]. A similar biphasic growth effect was observed in Swiss 3T3 cells expressing endogenous GRP-R, indicating that this effect was not unique to the NMB-R. These studies indicate that constitutive occupation of bombesin receptors present in high numbers (>50,000 receptors/cell) inhibits, rather than stimulates, growth. Growth inhibition may result from an overstimulation of a physiologic signal transduction pathway or inappropriate activation of a secondary signal transduction pathway which results in decreased proliferation. If the dose-response of the growth inhibition effect depends on high receptor number, lung cancer cells may be selected to express sufficient receptors for growth stimulation, but not inhibition, at the available ligand concentration. Future studies will be needed to determine the effects of receptor number on the growth response to a range of concentrations of bombesin-like peptides.

Three conserved protein kinase C phosphorylation sites are found in the intracellular 3 and 4 domains of 4/5 bombesin receptor sequences (i3 and i4, boxed regions, Fig. 3). The location of these potential sites of protein kinase C phosphorylation are in domains known to be critical for G-protein coupling in other G-protein coupled receptors, including the beta-adrenergic [38], muscarinic [38], and TSH receptors [39]. In a previous study of bombesin receptor function in small cell lung cancer cell line H345, the phorbol compound 12-myristate 13-acetate, a protein kinase C activator, attenuated bombesin-stimulated increases in intracellular Ca^{++} [40], indicating a role for protein kinase C phosphorylation in receptor desensitization. Zachary and coworkers [41] have reported that the early cellular responses following stimulation of the Swiss 3T3 GRP-R by ligand include activation of protein kinase C. Taken together, these observations suggest that bombesin receptors may be phosphorylated by protein kinase C at conserved sites in i3 and i4 after ligand activation, and that this post-translational modification may serve to desensitize the receptor by interfering with G-protein coupling. Analysis of the desensitization properties of receptors with mutations altering one or more of these three conserved sites should clarify the importance of this potential regulatory mechanism.

After expression of BRS-3 in *Xenopus* oocytes, the receptor is specifically activated by bombesin-like peptides, and not peptides from other neuropeptide families. However, in contrast to GRP-R and NMB-R, higher ligand concentrations (10^{-6} vs. 10^{-8} M) were required to elicit responses. Several explanations are possible for this difference between BRS-3 and the other two receptors: (1) a specific G-protein and/or auxillary protein(s) not available in the *Xenopus* oocyte may be needed for high affinity ligand binding. For example, the secretion receptor shows a ten-fold increase in ligand affinity when co-expressed with a specific G-alpha subunit, G_s, in COS cells [42]; (2) a critical post-translational modification that does not occur in *Xenopus* oocytes may be required for BRS-3 to bind ligands at high affinity binding; (3) BRS-3 mRNA or protein may be unstable in *Xenopus* oocytes, resulting in a low number of receptors expressed on the oocyte membrane; or (4) the mammalian bombesin-like peptide specific for BRS-3 is neither GRP nor NMB, and remains to be identified. Future efforts will be directed towards expressing BRS-3 in host cells other than *Xenopus* oocytes, including a panel of murine fibroblast cell lines. In addition, we are actively attempting to identify and characterize additional mammalian bombesin-like peptide ligands. Potential sources of these novel peptides include human lung carcinoma cell lines or testis, which are cell or tissue types that express BRS-3 [30].

No pre-existing pharmacologic study predicted the existence of BRS-3, the third human bombesin receptor to be cloned and characterized. The third exon of BRS-3 was initially identified and cloned as a 3.0 kb Eco RI human genomic DNA fragment that hybridized at low stringency to GRP-R and NMB-R cDNA probes at low stringency [30]. Several other fragments with similar hybridization properties were also noted, none of which encode exons of GRP-R, NMB-R, or BRS-3. These unexplained genomic DNA fragments may encode exons of additional human bombesin receptor genes that are not characterized at present. Future efforts will be directed towards the characterization of these potentially interesting sequences, which may provide a means for obtaining new cDNAs encoding additional human bombesin receptors which are likely to possess their own unique pharmacologic properties and pattern of expression. Given

the receptor heterogeneity observed to date, it is clear that further characterization of mammalian bombesin-like peptides and their receptors will provide additional useful information for the design of compounds and therapeutic regimens of potential importance in modulating bombesin-dependent growth in human neoplastic disease.

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