Biologically Active Recombinant Human Progastrin\textsubscript{6–80} Contains a Tightly Bound Calcium Ion* 

Received for publication, November 2, 2000

Graham S. Baldwin‡‡, Frédéric Hollande§, Zhiyu Yang‡, Yulia Karelina‡, Adrienne Paterson‡, Rosslyn Strange‡, Daniel Fourmy**, Greg Neumann‡‡, and Arthur Shulkes‡

From the ‡University Department of Surgery, Austin Hospital, Heidelberg, Victoria 3084, the §§Russell Grunwade School of Biochemistry, University of Melbourne, Melbourne, Parkville, Victoria 3052, and the ¶¶Department of Biochemistry, Latrobe University, Bundoora, Victoria 3083, Australia and the †Faculty de Pharmacie, Université de Montpellier, Montpellier, and the **INSERM U 151, CHU Rangéul, Toulouse, France

Evidence is accumulating that gastrin precursors may act as growth factors for the colonic mucosa in vitro. The aims of this study were to prepare recombinant human progastrin\textsubscript{6–80} and to investigate its structure and biological activities in vitro. Human progastrin\textsubscript{6–80} was expressed in \textit{Escherichia coli} as a glutathione S-transferase fusion protein. After thrombin cleavage progastrin\textsubscript{6–80} was purified by reverse phase high pressure liquid chromatography and characterized by radioimmunoassay, amino acid sequencing, and mass spectrometry. Assays for metal ions by atomic emission spectroscopy revealed the presence of a single tightly bound calcium ion. Progastrin\textsubscript{6–80} at concentrations in the pM to nM range stimulated proliferation of the conditionally transformed mouse colon cell line YAMC. The observations that progastrin\textsubscript{6–80} did not bind to either the cholecystokinin (CCK)-A or the gastrin/CCK-B receptor expressed in COS cells and that antagonists selective for either receptor did not reverse the proliferative effects of progastrin\textsubscript{6–80} suggested that progastrin\textsubscript{6–80} stimulated proliferation independently of either the CCK-A or the gastrin/CCK-B receptor. We conclude that recombinant human progastrin\textsubscript{6–80} is biologically active and contains a single calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and proinsulin, this is the first report of selective, high affinity binding of metal ions to a prohormone.

Biologically activity, glycine-extended gastrin\textsubscript{17} has been shown to stimulate the proliferation of several cell lines (2–4). Progastrin itself appears to act as a growth factor for normal colon, because transgenic mice expressing progastrin in the liver have increased concentrations of serum progastrin and a hyperplastic colonic mucosa (5). In addition, the observation of increased numbers of aberrant crypt foci (6) and tumors (7) in the colonic mucosa of transgenic mice overexpressing progastrin in comparison with wild-type mice following treatment with azoxymethane suggests that progastrin may act as a co-carcinogen in the development of colorectal carcinoma. However, the possibility should be borne in mind that progastrin, or a breakdown product, might have been acting indirectly on a tissue other than the colonic mucosa to release a second growth factor responsible for the effects observed in the colon.

The possibility that colorectal carcinoma cells might utilize progastrin or progastrin-derived peptides as autocrine growth factors has recently received considerable attention (8). The autocrine model predicts that a cell synthesizes a particular growth factor, which, after release into the surrounding medium, binds to specific receptors on the surface of the same cell and stimulates the proliferation of that cell. The observation that expression of antisense gastrin mRNA inhibits proliferation of colon-derived cell lines in vitro and in vivo (3, 4) provides strong evidence that progastrin or progastrin-derived peptides may act as autocrine growth factors in colorectal carcinoma. As predicted by the autocrine model, most colon carcinomas and derived cell lines synthesize gastrin mRNA and progastrin-derived peptides (see Ref. 8 for review) and increased concentrations of progastrin-derived peptides have been detected in the sera of patients with colorectal carcinoma (9). However, the identity of gastrin receptors on colorectal carcinomas is still unclear (see Ref. 8 for review).

Experiments on the role of progastrin and its receptors in the development of colorectal carcinoma have been limited by the scarcity of the prohormone. Small amounts of progastrin\textsubscript{1–80} (less than 1 nmol/gm tissue) have been isolated from human tissues (10), but the largest progastrin-derived peptide available in bulk to date via organic synthesis is progastrin\textsubscript{20–71} (10). The related prohormone procholecystokinin (pro-CCK)\textsuperscript{1} has been expressed with an N-terminal histidine tag and purified from baculovirus-infected insect cells (11). We have now developed a method for expression and purification of progastrin\textsubscript{6–80} from \textit{Escherichia coli}, to test directly its biological activities on colon-derived cell lines in vitro, to measure its affinity for gastrin and CCK receptors, and to investigate its structure.

Gastrin is a classical gut peptide hormone that was identified originally as a stimulant of gastric acid secretion. Like many other peptide hormones, gastrin is synthesized as a large precursor molecule of 101 amino acids (Fig. 1), which is converted to progastrin (80 amino acids) by cleavage of the N-terminal signal peptide. Progastrin is processed further by endo- and carboxypeptidases and by C-terminal amidation to yield the final end products glycine-extended gastrin\textsubscript{17} and amidated gastrin\textsubscript{17} (1). Although amidated gastrins were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed at the following address: Dept. of Surgery, Austin Campus, A&RMC, Studley Rd., Heidelberg, Victoria 3084, Australia. Tel.: 613-9496-5592; Fax: 613-9458-1650; E-mail: g.baldwin@surgeyaustin.unimelb.edu.au.

* This work was supported in part by the Austin Hospital Medical Research Foundation and by Grants 940924 and 980625 (to G. B.) and 960258 and 114123 (to A. S.) from the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed at the following address: Dept. of Surgery, Austin Campus, A&RMC, Studley Rd., Heidelberg, Victoria 3084, Australia. Tel.: 613-9496-5592; Fax: 613-9458-1650; E-mail: g.baldwin@surgeyaustin.unimelb.edu.au.

1 The abbreviations used are: CCK, cholecystokinin; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline.
The supernatant was transferred to a new tube, and Triton X-100 was added. The samples were subjected to circular dichroism (12) or NMR spectroscopy (14). In aqueous solution because no binding was detected by either circular dichroism (12) or NMR spectroscopy (14). In trilfluoroethanol the dissociation constants for binding of Ca^{2+} to the three sites in [Nle^{11}]-gastrin_{17} and [Nle^{15}]-gastrin_{17} was observed in trilfluoroethanol (12–13), but the affinities are presumably considerably lower in aqueous solution because no binding was detected by either circular dichroism or NMR spectroscopy. The structure of the GST-progastrin fusion protein is presented in the structure shown in colored letters with progastrin sequences in capital letters and linker sequences in lowercase letters. Numbering commences at the N terminus of mature progastrin (10). Thrombin cleavage sites are indicated by vertical arrows.

Previous structural studies on progastrin-derived peptides have been limited to investigation of the conformation of gastrin, and shorter fragments by circular dichroism and ultraviolet and NMR spectroscopy (Refs. 12–14 and references therein). Binding of three Mg^{2+} or Ca^{2+} ions to human [Nle^{11}]-gastrin_{13} and [Nle^{15}]-gastrin_{17} was observed in trilfluoroethanol (12–13), but the affinities are presumably considerably lower in aqueous solution because no binding was detected by either circular dichroism or NMR spectroscopy (14). In trilfluoroethanol the dissociation constants for binding of Ca^{2+} to the three sites in [Nle^{11}]-gastrin_{17} were $K_1 = 0.29 \mu M$, $K_2 = 0.29 \mu M$, and $K_3 = 7.1 \mu M$. To determine whether the N- and C-terminal extensions of progastrin_{5–80} increased its affinity for metal ions, samples of recombinant human progastrin_{5–80} were analyzed by inductively coupled plasma atomic emission spectroscopy. Here we report that progastrin_{5–80} is biologically active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion. With the exception of the well known zinc-dependent polymerization of insulin and prooinsulin (15), this is the first report of selective, active and contains a single tightly bound calcium ion.
once in PBS, and incubated for 1 h with a mouse anti-bromodeoxyuridine antibody. After three rinses in PBS, the cells were incubated with FITC-labeled goat anti-mouse IgG for 30 min. After three further rinses the coverslips were mounted on slides with cytofluor and observed on a fluorescence microscope.

Transient Transfection of COS Cells—COS cells were transiently transfected by the DEAE-dextran method as described previously (21). One day before transfection, 0.7–1.0 × 10^6 COS cells were seeded in 10-cm plates in Dulbecco's modified Eagle's medium and grown in 5% CO_2 such that on the day of transfection the cells were 60% confluent. On the day of transfection, a DNA/DEAE-dextran solution was prepared by dropwise addition of 0.5 ml 2 mg/ml DEAE-dextran in PBS to 0.5 ml of 0.1% glucose in PBS containing 3.5 mg/ml pRFNeo plasmid DNA encoding either the human CCK-A or the human CCK-B receptor (22). The medium was aspirated, and the cells were washed once with PBS and gently rocked at 37 °C for 20 min in the DNA/DEAE-dextran solution. The solution was then replaced with 10 ml of 100 μM chloroquine, and the cells were incubated at 37 °C for 3.5 h. After incubation, the solution was aspirated, and the cells were washed twice with serum-free Dulbecco's modified Eagle's medium with 10% fetal calf serum overnight. On the next day, the transfected cells were dislodged with 0.02% EDTA, replated onto a 24-well dish (20,000–50,000/well) and grown for a further 48 h prior to the receptor binding assay.

Receptor Binding Assays—Binding of progastrin to either the human CCK-A receptor or the human gastrin/CCK-B receptor was measured by competition for 125I-labeled Bolton and Hunter CCK binding as described by Kopin and coworkers (23). Transfected COS7 cells were grown to 60–70% confluence as described above, washed once with PBS, and then incubated for 80 min at 37 °C in 150 μl of Dulbecco's modified Eagle's medium containing [125I]CCK, (50,000 cpm, 14.5 fmol; American Pharmacia Biotech), 150 μM phenylmethylsulfonyl fluoride, 0.05% bacitracin, and 0.1% BSA. Cells were then washed twice with PBS and lysed with 300 μl of 1 M NaOH. Lysates were counted in a γ-counter (LKB-Wallac, Turku, Finland) at 77% efficiency. Estimates of IC_{50} values and of the levels of [125I]CCK bound in the absence of competitor were fitted as previously described (24).

Statistics—Results are expressed as the means ± S.E., except where otherwise stated. Parametric and nonparametric data sets were analyzed by one-way analysis of variance and by Kruskal-Wallis one-way analysis of variance on ranks, respectively. If there was a statistically significant difference in the mean or median values of each set, the values were individually compared with the control value by Dunnett's or Dunn's methods, respectively. Differences with p values of < 0.05 were considered significant.

RESULTS

Expression of Human Progastrin in E. coli—Human progastrin was expressed in E. coli as a fusion protein with glutathione S-transferase (Fig. 2). The fusion protein was isolated by binding to glutathione-agarose beads according to Frangioni and Neel (19). Recombinant human progastrin was cleaved from the fusion protein bound to glutathione-agarose beads by treatment with thrombin (Fig. 2).

Purification and Characterization of Recombinant Human Progastrin—Recombinant human progastrin was purified by reverse phase HPLC (Fig. 3). The absorbance peak at fraction 21–22 matched very well with the peak of immunoreactivity observed with antiserum 1137, which was raised against an undecapeptide consisting of the C-terminal undecapeptide of progastrin (residues 71–80) with an additional tyrosine residue at the N terminus for iodination (9). The conclusion that the recombinant human progastrin contained the C terminus of progastrin was confirmed by electrospray ionization mass spectrometry. The molecular mass of HPLC-purified recombinant human progastrin was 8427.1 ± 0.7 Da, which is in excellent agreement with the mass of 8427.1 Da predicted for human progastrin. The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6–13 of human progastrin. Because the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6.

Metal Analyses—Previous reports have indicated that human [Nle^{11}]-gastrin_{97} and [Nle^{13}]-gastrin_{97} bind three Mg^{2+} or Ca^{2+} ions in trifluoroethanol (12–13). The presence of metal ions in recombinant human progastrin was investigated by inductively coupled plasma atomic emission spectroscopy. The analysis revealed that recombinant human progastrin contained 8427.1 ± 0.7 Da, which is in excellent agreement with the mass of 8427.1 Da predicted for human progastrin. The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6–13 of human progastrin. We conclude that the HPLC-purified recombinant human progastrin consists of residues 6–80 inclusive of human progastrin. Because the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6.

Metal Analyses—Previous reports have indicated that human [Nle^{11}]-gastrin_{97} and [Nle^{13}]-gastrin_{97} bind three Mg^{2+} or Ca^{2+} ions in trifluoroethanol (12–13). The presence of metal ions in recombinant human progastrin was investigated by inductively coupled plasma atomic emission spectroscopy. The analysis revealed that recombinant human progastrin contained 8427.1 ± 0.7 Da, which is in excellent agreement with the mass of 8427.1 Da predicted for human progastrin. The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6–13 of human progastrin. We conclude that the HPLC-purified recombinant human progastrin consists of residues 6–80 inclusive of human progastrin. Because the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6.

Metal Analyses—Previous reports have indicated that human [Nle^{11}]-gastrin_{97} and [Nle^{13}]-gastrin_{97} bind three Mg^{2+} or Ca^{2+} ions in trifluoroethanol (12–13). The presence of metal ions in recombinant human progastrin was investigated by inductively coupled plasma atomic emission spectroscopy. The analysis revealed that recombinant human progastrin contained 8427.1 ± 0.7 Da, which is in excellent agreement with the mass of 8427.1 Da predicted for human progastrin. The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6–13 of human progastrin. We conclude that the HPLC-purified recombinant human progastrin consists of residues 6–80 inclusive of human progastrin. Because the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6.

Metal Analyses—Previous reports have indicated that human [Nle^{11}]-gastrin_{97} and [Nle^{13}]-gastrin_{97} bind three Mg^{2+} or Ca^{2+} ions in trifluoroethanol (12–13). The presence of metal ions in recombinant human progastrin was investigated by inductively coupled plasma atomic emission spectroscopy. The analysis revealed that recombinant human progastrin contained 8427.1 ± 0.7 Da, which is in excellent agreement with the mass of 8427.1 Da predicted for human progastrin. The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6–13 of human progastrin. We conclude that the HPLC-purified recombinant human progastrin consists of residues 6–80 inclusive of human progastrin. Because the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6.

Metal Analyses—Previous reports have indicated that human [Nle^{11}]-gastrin_{97} and [Nle^{13}]-gastrin_{97} bind three Mg^{2+} or Ca^{2+} ions in trifluoroethanol (12–13). The presence of metal ions in recombinant human progastrin was investigated by inductively coupled plasma atomic emission spectroscopy. The analysis revealed that recombinant human progastrin contained 8427.1 ± 0.7 Da, which is in excellent agreement with the mass of 8427.1 Da predicted for human progastrin. The N-terminal amino acid sequence of HPLC-purified recombinant human progastrin determined by Edman analysis was SQQPDAPL, which corresponded precisely to residues 6–13 of human progastrin. We conclude that the HPLC-purified recombinant human progastrin consists of residues 6–80 inclusive of human progastrin. Because the N-terminal sequence of human progastrin is SWKPRSQQPDAPL, it appears that thrombin has cleaved the peptide bond between the arginine residue at position 5 and the serine residue at position 6.
Recombinant human progastrin was synthesized as a fusion protein with GST (Fig. 2). This peptide was purified from progastrin8427.1 expected for human progastrin 6–80. This peptide would be generated by cleavage between Arg9 and Ser10 in the progastrin sequence Trp-Lys-Pro-Arg-Ser-Gln, which is consistent with the preferred thrombin recognition sequence P4-P3-Pro-Arg/Lys-P1′-P2′, where P3 and P4 are hydrophobic amino acids, and P1′ and P2′ are nonacidic amino acids (27). Isolation of progastrin6–35 and progastrin9–35 from a human gastrinoma by Reeve and co-workers (28) had previously revealed that the signal peptide of pre-progastrin contains a tightly bound calcium ion. Samples of recombinant human progastrin6–80 contained 1.06 ± 0.08 mol calcium ions/mol (mean ± S.E., n = 3) (Fig. 4). No other cations were detected consistently. The calcium ion was not removed by extensive dialysis at pH 7.6 against 10 mM EDTA (data not shown), or by treatment with 8 M urea containing 10 mM EDTA (data not shown), or by extensive dialysis at pH 5.5 against 100 mM EDTA (data not shown).

Proliferation Studies—Recombinant human progastrin6–80 stimulated proliferation of the conditionally transformed mouse colon cell line YAMC in a dose-dependent manner with maximal stimulatory effects seen in the range 10 to 100 nM (Fig. 5A). The stimulatory effect of recombinant human progastrin6–80 on YAMC cells was unaffected by either the CCK-A receptor-selective antagonist L364,718 or the gastrin/CCK-B receptor-selective antagonist L365,260 at concentrations as high as 10 μM (Fig. 5B). Gastrin17gly also stimulated proliferation of YAMC cells in the concentration range 1 pM to 1 nM, as has been reported previously with a colorimetric assay (4).

Receptor Binding—Studies of progastrin binding were confined to CCK-A and gastrin/CCK-B receptors, which have both been fully characterized at the nucleotide sequence level. Binding of recombinant human progastrin6–80 to either the human CCK-A or human gastrin/CCK-B receptor was investigated by competition for the binding of [125I]CCK8 to transiently transfected COS cells as described under "Experimental Procedures." Recombinant human progastrin6–80 had no effect on the binding of [125I]CCK8 to the gastrin/CCK-B receptor even at concentrations as high as 100 nM (Fig. 6) and consistently stimulated the binding of [125I]CCK8 to the CCK-A receptor. CCK8 and gastrin17 were used as positive controls for measurement of binding to the CCK-A and gastrin/CCK-B receptors. The measurement of stimulatory effect of recombinant human progastrin6–80 on CCK-A or human gastrin/CCK-B receptor was investigated by measurement of absorbance at 280 nm. Error bars represent the S.E.; similar results were obtained in a second experiment.

In this paper the production and purification of recombinant human progastrin6–80 from E. coli is reported for the first time. Progastrin was synthesized as a fusion protein with GST (Fig. 1) and partially purified by utilizing the affinity of GST for glutathione-agarose (Fig. 2). After treatment of the bound fusion protein with thrombin, progastrin6–80 was released into the supernatant, which was separated from the glutathione-agarose-bound GST by centrifugation. Final purification of the supernatant by reverse phase HPLC resulted in preparations of recombinant human progastrin that were homogeneous by gel electrophoresis (Fig. 2) and mass spectrometry.

The molecular mass of recombinant human progastrin determined by electrospray ionization mass spectrometry was 8427.1 ± 0.7 Da, in excellent agreement with the value of 8427.1 Da expected for human progastrin6–80. This peptide would be generated by cleavage between Arg9 and Ser10 in the progastrin sequence Trp-Lys-Pro-Arg-Ser-Gln, which is consistent with the preferred thrombin recognition sequence P4-P3-Pro-Arg/Lys-P1′-P2′, where P3 and P4 are hydrophobic amino acids, and P1′ and P2′ are nonacidic amino acids (27). Isolation of progastrin6–35 and progastrin9–35 from a human gastrinoma by Reeve and co-workers (28) had previously revealed that the signal peptide of pre-progastrin was 21 amino acids long and that an additional cleav-
Biologically Active Recombinant Progastrin

7795

**Fig. 6. Recombinant human progastrin does not bind to the gastrin/CCK-B receptor.** Binding of \[^{125}\text{I}]\text{CCK}_\text{A} (30 \text{ pm}, 20,000 \text{ cpm}) to COS cells expressing the human CCK-A (A) or gastrin/CCK-B (B) receptors was measured in the presence of increasing concentrations of recombinant human progastrin 1–80 (closed squares) as described under “Experimental Procedures.” Binding in the presence of increasing concentrations of Ca\(^{2+}\) (A, open circles) and gastrin 1–35 (B, open squares) was measured as a control. Values are expressed as percentages of the value obtained in the absence of competitor. Points are the means ± S.E. of triplicates from three experiments, and the line of best fit was obtained by nonlinear regression to a single site model as described previously (34). The values for IC\(_{50}\) and for the predicted ordinate intercept were 12 ± 5 nm and 84 ± 6% for the CCK-A receptor and 36 ± 15 nm and 81 ± 7% for the gastrin/CCK-B receptor, respectively. Statistical significance compared with controls without unlabeled peptide was assessed as described under “Experimental Procedures.” *p < 0.05. Progastrin \(_{6-80}\) did not compete with \[^{125}\text{I}]\text{CCK}_\text{A} for binding to the gastrin/CCK-B receptor (B). Binding of \[^{125}\text{I}]\text{CCK}_\text{A} to the CCK-A receptor (A) was increased in the presence of progastrin \(_{6-80}\).**

---

The affinity of progastrin \(_{6-80}\) for the calcium ion is high, because the metal ion was not removed by extensive dialysis against EDTA at pH 7.6, or 5.5, or by treatment with 8 M urea in the presence of EDTA. Binding of three Mg\(^{2+}\) or Ca\(^{2+}\) ions to human [Nle\(^{11}\)]-gastrin \(_{17}\) and [Nle\(^{15}\)]-gastrin \(_{17}\) in trifluoroethanol has been reported previously (12–13), but no binding has been detected in aqueous solution by circular dichroism (12) or by NMR spectroscopy (14). The dissociation constants for binding of Ca\(^{2+}\) to the three sites in [Nle\(^{15}\)]-gastrin \(_{17}\) in trifluoroethanol were \(K_1 = 0.29 \mu\text{M},\; K_2 = 0.29 \mu\text{M},\) and \(K_3 = 7.1 \mu\text{M.}\) The apparent increase in affinity for Ca\(^{2+}\) ions between human [Nle\(^{15}\)]-gastrin \(_{17}\) and human progastrin \(_{6-80}\) indicates either that the structure of the pentaglutamate sequence of gastrin \(_{13}\) is altered by the addition of the N- and C-terminal extensions of progastrin \(_{6-80}\), with a consequent increase in affinity, or that a new binding site is created by the additional amino acids. The decrease in the stoichiometry of calcium binding from 3 in gastrin \(_{17}\) to 1 in progastrin \(_{6-80}\) favors the latter explanation. The observation that there are several acidic residues that are conserved across species (30) in both N- and C-terminal extensions is also consistent with the existence of a different binding site. However, the absence of previously described motifs such as the E-F hand from both N- and C-terminal extensions suggests that the calcium binding site may be different from known binding sites.

A number of possible roles for the tightly bound Ca\(^{2+}\) ion can be envisaged. Firstly, the Ca\(^{2+}\) ion might contribute to the thermostability of progastrin, as has been reported previously for binding of Ca\(^{2+}\) ions to trypsin (31). Secondly, the Ca\(^{2+}\) ion might alter the ability of progastrin to polymerize, as has been reported previously for the binding of Zn\(^{2+}\) ions to proinsulin (29). For example, during biosynthesis and storage in the pancreatic \(\beta\)-cell proinsulin assembles into dimers, which in the presence of Zn\(^{2+}\) or other divalent metal ions further assemble into hexamers. Thirdly, the Ca\(^{2+}\) ion might enhance the solubility of progastrin, as has been reported previously for the binding of Zn\(^{2+}\) ions to proinsulin (32). Fourthly, the Ca\(^{2+}\) ion might redirect the processing of progastrin by preventing cleavage at some dibasic sites, in the same way that phosphorylation of Ser\(^{75}\) prevents cleavage of the Arg\(^{73}\)–Ser\(^{74}\) bond (33).

The observation that the calcium remained bound to progastrin even after extensive dialysis at pH 5.5 (data not shown), which is the pH within the secretory granule where processing occurs (34), is consistent with a role in processing. The fact that amidated and nonamidated gastrins act on different receptors to generate different effects (8) suggests that such modification of the processing pathways could profoundly effect biological activity. Experimental testing of all of the above hypotheses will require the development of methods for removal of the Ca\(^{2+}\) ion from progastrin.

Proliferation of the conditionally transformed mouse colon cell line YAMC (16) was stimulated by concentrations of recombinant human progastrin \(_{6-80}\) in the pm to nm range (Fig. 5A). As well as demonstrating that recombinant human progastrin \(_{6-80}\) is correctly folded when synthesized as a fusion protein in E. coli and is not denatured during purification, the proliferation data provide the first evidence that progastrin has a direct effect on cells of colonic origin. Previous reports that progastrin acts as a growth factor for normal colon in transgenic mice expressing progastrin in the liver (5) and that such mice have increased numbers of aberrant crypt foci (6) and tumors (7) in the colonic mucosa following treatment with azoxymethane in comparison with wild-type mice may be subjected to the criticism that the observed effects on the colonic mucosa may not reflect a direct effect of progastrin itself. For example progastrin synthesized from the liver transgene could have been acting on an unidentified cell type, in a tissue other than the colon, to release a second hormone active on colonic cells. In addition, in the currently available mouse models the
duration of exposure to progastrin-derived peptides and the serum concentrations of progastrin-derived peptides were not controlled (5, 6), so that the transgenic mice were exposed to widely varying concentrations of progastrin-derived peptides both in utero and throughout their adult life. The stimulation of YAMC cell proliferation in the presence of progastrin_{6–80} reported herein (Fig. 5) clearly demonstrates that progastrin_{6–80} itself has short term direct effects on cells of colonic origin.

Attempts to define the binding properties of the progastrin receptor responsible for mediating the proliferative effects of progastrin on YAMC cells have been unsuccessful. As yet we have been unable to iodinate progastrin reproducibly by either the chloramine T or iodogen methods, possibly because the single tyrosine residue is buried within the progastrin structure. We have succeeded in labeling the GST-progastrin fusion protein, presumably on one or more of the 14 tyrosines in the GST sequence. However the presence of the GST appears to prevent receptor binding, because no binding of the iodinated fusion protein to YAMC cells has been detected.

The following observations suggest that the biological effects of progastrin are not mediated by either the CCK-A or gastrin/CCK-B receptor. Firstly recombinant human progastrin_{6–80} does not bind to the gastrin/CCK-B receptor at concentrations as high as 100 nM (Fig. 6). Secondly the stimulatory effect of recombinant human progastrin on YAMC cells was unaffected by either the CCK-A receptor-selective antagonist L364,718 or the gastrin/CCK-B receptor-selective antagonist L365,260, at concentrations as high as 10 µM (Fig. 5B). Thirdly previous studies have not detected high affinity binding sites for 125I-gastrin_{17} on YAMC cells, and amidated gastrin_{17} has no effect on their proliferation (4).

Surprisingly, binding of 125I-CCK_{8} to the CCK-A receptor was consistently higher in the presence of recombinant human progastrin_{6–80} (mean percentage of control ± S.E. = 144 ± 7) (Fig. 6). One possible explanation for the increase is that progastrin_{6–80} is binding to the CCK-A receptor at a site distinct from the CCK binding site and that the binding of progastrin_{6–80} increases the affinity of the CCK binding site for 125I-CCK_{8}. The absence of detectable competition between progastrin_{6–80} and 125I-CCK_{8} for binding to either the CCK-A or gastrin/CCK-B receptor is in agreement with previous reports that removal of the C-terminal amide group from CCK (35) or gastrin (36) results in a substantial reduction in affinity for the CCK-A and gastrin/CCK-B receptor, respectively.

The receptor binding data presented herein have significant implications for our understanding of the mechanism by which progastrin stimulates growth of the colonic mucosa. The inability of the CCK-A and gastrin/CCK-B receptors to recognize progastrin clearly indicates that neither receptor is involved in the hyperplasia (5) or enhanced development of aberrant crypt foci (6) or tumors (7) observed in the colonic mucosa of mice rendered hyperprogastrinemic by expression of a progastrin transgene in the liver. Experiments are underway to determine whether or not other high affinity receptors selective for progastrin are present in the normal colonic mucosa and to define the signaling pathways involved in the proliferative effects.

In summary, this paper describes the first synthesis of recombinant human progastrin_{6–80}. The observation of progastrin-dependent proliferation of the mouse colonic line YAMC confirms that the recombinant peptide is biologically active and is consistent with the previously reported proliferative effects of endogenous progastrin on the colonic mucosa of transgenic mice (5). The observations that progastrin does not bind to either the CCK-A or the gastrin/CCK-B receptors and that antagonists selective for either the CCK-A or the gastrin/CCK-B receptor do not affect progastrin-induced proliferation indicate that the proliferative effects of progastrin are independent of either the CCK-A or the gastrin/CCK-B receptors. Recombinant human progastrin_{6–80} contains a single calcium ion, but the high binding affinity has so far prevented analysis of the role, if any, of the calcium ion in biological activity. With the exception of the well known zinc-dependent polymerization of insulin and proinsulin (15), this is the first report of selective, high affinity binding of metal ions to a prohormone. It is anticipated that recombinant human progastrin_{6–80} will be an essential tool with which to investigate the biological effects of progastrin in vivo, the nature of the receptors involved, the role of the tightly bound calcium ion in biological activity, and the structure of progastrin itself.

Acknowledgments—We gratefully thank Rosemary Condron (Department of Biochemistry, Latrobe University) for the amino acid sequencing, Dr. Peter Curtis (Commonwealth Scientific and Industrial Research Organisation Division of Manufacturing Science and Technology) for metal analyses, and Professor R. J. Wettenhall (Russell Grimwade School of Biochemistry, University of Melbourne) for many helpful discussions.

REFERENCES

1. Dockray, G. J., Varro, A., and Dimaline, R. (1996) Physiol. Rev. 76, 767–788
2. Seva, C., Dickinson, C. J., and Yamada, T. (1994) Science 265, 410–412
3. Singh, P., Velasco, M., Given, R., Wargovich, M., Varro, A., and Wang, T. C. (2000) Am. J. Physiol. 278, G390–G399
4. Singh, P., Velasco, M., Given, R., Varro, A., and Wang, T. C. (2000) Gastroentorology 119, 162–171
5. Baldwin, G. S., and Shulkes, A. (1998) Gut 42, 581–584
6. Ciccotosto, G. D., MeLeish, A., Hardy, K. J., and Shulkes, A. (1995) Gastroenetrology 109, 1142–1153
7. Rehfeld, J. F., and Johnsen, A. H. (1994) Eur. J. Biochem. 223, 765–773
8. Wang, Y., Yunn, L., and Beinfeld, M. C. (1997) Peptides. 18, 1295–1299
9. Peggion, E., Mammi, S., Palumbo, M., Moroder, L., and Wunsch, E. (1983) Biopolymers 23, 2443–2457
10. Peggion, E., Mammi, S., Palumbo, M., Moroder, L., and Wunsch, E. (1984) Biopolymers 23, 1259–1259
11. Torda, A. E., Baldwin, G. S., and Norton, R. S. (1985) Biochemistry 24, 1720–1727
12. Blundell, T. L., Dodson, G. G., Dodson, E., Hodgkin, D. C., and Vijayan, M. (1971) Recent Prog. Horm. Res. 27, 1–40
13. Whitehead, R. H., VanBodegom, P. E., Nobe, M. D., Atlitiosis, J., and Pats, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 587–591
14. Boel, E., Vuvst, J., Norris, F., Norris, K., Wind, A., Rehfeld, J. F., and Markert, K. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2866–2869
15. Smith, D. B., and Johnson, K. S. (1988) Biochemistry 27, 179–187
16. Gallagher, S. R. (1988) in Current Protocols in Protein Science (Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T., eds) pp. 10.11–10.12. John Wiley & Sons, New York
17. Mamantaditos, T., and Baldwin, G. S. (1994) Biochem. Biophys. Res. Commun. 201, 1382–1389
18. Kennedy, K., Escrieut, C., Dupreane, M., Clerc, P., Vaysse, N., and Fourmy, D. (1995) Biochem. Biophys. Res. Commun. 213, 845–852
19. Kopin, M. G., and Shulkes, A. (1995) Diabetologia 38, 174–182
20. Derewenda, U., Derewenda, Z., Dodson, G. G., and Hubbard, R. E., and Varro, F. (1998) J. Biol. Chem. 273, 20411–20414
21. Derewenda, U., Derewenda, Z., Dodson, G. G., and Hubbard, R. E., and Varro, F. (1998) J. Biol. Chem. 273, 20411–20414