A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa

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Martin, Terence E., C. Thomas Powell, Zunde Wang, Somnath Bhattacharyya, Margaret M. Walsh-Reitz, Kan Agarwal, and F. Gary Toback. A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa. Am J Physiol Gastrointest Liver Physiol 285: G332–G343, 2003; 10.1152/ajpgi.00453.2002.—Human and pig cDNAs for a novel stomach protein, the product of a gene expressed at high levels specifically in cells of the antrum mucosa, have been characterized. The general exon/intron structure of the genomic DNA is conserved in humans and mice. The predicted protein sequences of the human and mouse mRNAs contain 185 and 184 amino acids, respectively. The protein isolated from pig antral extracts has an NH2 terminus consistent with cleavage of a 20-amino acid signal peptide. Human cDNA was expressed in E. coli to generate a protein antigen for antibody production. The antibodies detected polypeptides of ~18 kDa in antrum extracts from all mammalian species tested. Immunocytochemistry located antrum mucosal protein (AMP)-18 to surface mucosal cells of the mouse antrum and, specifically, to secretion granules, suggesting that it is cosecreted with mucins. Antrum extracts and recombinant human AMP-18 exhibit growth-promoting activity on epithelial cells that can be blocked by the specific antisera. We suggest that AMP-18 is a “gastrokine” that maintains the integrity of the gastric mucosal epithelium.

Growth factor; stomach; epithelium

ISOLATION OF THE CDNA CLONE encoding gastrin was one of the earliest triumphs of molecular biology (28). Both the restricted expression of gastrin to cells of the gastric antrum mucosa and the capacity of this peptide hormone to regulate acid secretion by, and stimulate growth of the stomach mucosa, are now well known (3, 19). During the initial screening of gastrin clones, another mRNA whose expression appeared to be specific for cells of the gastric antrum was found, but not characterized. Later, when the open-reading frame (ORF) of this RNA was studied, it was found to be highly conserved among pig, humans, and mouse, but predicted a novel protein of no obvious function (17). Here we present a series of studies in which molecular and cell biological characterization of this antral-specific cDNA clone was carried out with the goal of defining the physiological role of the protein product. The results describe an apparently novel antral mucosal protein (AMP) that is ~18 kDa in size, herein called AMP-18. This protein is found in mucous secretion granules in gastric surface cells and appears to function as an epithelial cell mitogen.

MATERIALS AND METHODS

Initial cDNA clones. An antrum-specific pig cDNA clone (no. 1888) encoding AMP-18 was isolated by screening a full-length cDNA library synthesized from porcine antral mucosal poly(A)-enriched RNA (17) with antrum-specific sequence band B, as previously described (28). Band B was a partial-length cDNA synthesized specifically from porcine antral mucosal tissue RNA, by using a deoxydodecanucleotide primer complementary to gastrin mRNA. An homologous human clone (no. 1666) was isolated from a human antral mucosal cDNA library by screening with the pig 1888 clone. Both clones were sequenced by dyeoxy chain termination sequencing of M13 subclones and Maxam-Gilbert sequencing of restriction fragments (17). The human clone has subsequently been resequenced in the Cancer Research Center Sequencing Core Facility (University of Chicago) by using the dyeoxy method in the ABI Prism model 377. The Core Facility also carried out all other sequence determinations described below. AMP-18 cDNA sequences for pig (AY139185) and human (AY139184) are available at GenBank.

Northern blot confirmation of tissue-specific RNA expression of AMP-18. An internally [32P]phosphate-labeled probe was prepared by the primer extension-restriction digest method (6), by using an M13 mp11 subclone containing a 628 nucleotide fragment of the pig 1888 cDNA sequence. The probe contained the M13 primer and the first 34 nucleotides of the M13 mp11 minus strand downstream of the primer, in addition to the 628 nucleotide pig 1888 sequence. The M13 sequence did not cause any background radioactivity in the Northern hybridizations, so that its removal was not necessary. For Northern blots, total cellular RNA samples were prepared from mucosal tissue obtained from specific regions of the pig gastrointestinal (GI) tract by a modification of the method of Chirgwin et al. (2). RNA samples were denatured with glyoxal, electrophoresed on 1.5% agarose gels, transferred to nitrocellulose sheets, and hybridized with the probe as described by Thomas (23). About 1 × 106 counts per minute (cpm) of probe (specific activity ~1 × 108 cpm/mg) were used per milliliter of hybridization buffer per 3 cm2 of nitrocellulose.

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Genomic DNA amplification, cloning, and sequencing. The sequence determined for the human cDNA clone was used as the basis for designing PCR primers to investigate the structure of genomic DNA. Human genomic DNA was purchased from BD Biosciences Clontech (Palo Alto, CA). Mouse genomic DNA was prepared from the spleen of a male C57BL/6J mouse. PCR reactions were performed with the Expand High-Fidelity PCR System (Roche Diagnostics, Indianapolis, IN) by using 100 ng of template, 150 mM dNTPs, and 100 nM of primers in 50-ml reactions. The amplifications were performed in a GeneAmp PCR System 9700 (Perkin Elmer Life Sciences, Boston, MA) under the following conditions: 94°C for 3 min, followed by 20 cycles of 94°C for 30 s, 65°C (with 0.5°C decrease for every cycle) for 30 s, and 68°C for 2–10 min, depending on the length of the product, and then 15 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2–10 min. After the final cycle, samples were incubated at 68°C for 10 min and then placed at 4°C. The amplified products were cloned into pCR-BluntII-TOPO cloning system (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. DNA was prepared with Qiagen spin columns (Qiagen, Valencia, CA), and the amplified products were cloned with pCR-BluntII-TOPO (Invitrogen, Beverly, MA) was used to circularize the single-strand cDNA. Two gene-specific primers were prepared in opposite orientation to amplify the circular molecules, and the resulting products were cloned and sequenced to determine the transcription initiation site.

Sequence analysis methods. The compiled sequences for the human and mouse genes were analyzed for splice sites and promoter sequences by web-based programs (www.fruitfly.org/seq_tools/). DNA and protein sequence alignments were performed by using the University of Wisconsin GCG Package. BLAST (www.ncbi.nlm.nih.gov) was used to search for similarities with sequences in GenBank databases.

Mapping of the mouse gene. To map the mouse gene to its chromosomal location, we PCR amplified and sequenced a portion of the AMP-18 gene of a wild mouse strain, M. spreptus. A RsaI restriction polymorphism between the wild strain and C57BL/6J was found in 253-bp region segments comprising the exon 4/intron 4 junction region. RsaI was, therefore, used in typing the 188 progeny of the Jackson Laboratory Mouse Backcross Mapping Panels (18); the latter were obtained and typed by restriction digestion of the PCR products. The segregation pattern of the gene with mapped RsaI polymorphism was then detected to localize it to a specific chromosomal region between two markers. The genetic distances of the gene to the markers were calculated with the number of crossovers between the gene, and each marker was divided by the total number of meiotic events.

Expression of full-length recombinant human pre-AMP-18 protein and antibody production. For initial expression of the human protein in E. coli, an amplified sequence was generated from clone no. 1666 using PCR primers containing NcoI and HindIII sites. This construct was based on the presumption that the second ATG in the full-length sequence was the initiation codon (subsequent experiments, including those in RESULTS, support this assignment). The amplified sequence was inserted into the respective sites for those enzymes in the plasmid pTI(CCAAT/enhancer-binding protein), which contained a His6 tag upstream of the insertion site (29). The coding region of the construct would, therefore, be expected to express a protein containing a His6 tag linked to the N-terminus of the human protein. This pE1666 vector was used to transform BL21 (DE3)pLysS E. coli cells, which were grown to an optical density of 0.6 at 600 nm, and then induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside. Two hours after induction, the cells were harvested by centrifugation. The cells were lysed, the lysate was centrifuged, and proteins in the insoluble fraction were separated by electrophoresis on an SDS-polyacrylamide gel (12.5%) gel, which was then stained with Coomassie blue (0.1%). Attempts to obtain the recombinant protein in soluble form in the absence of SDS were unsuccessful. For the production of antibodies, the easily visible isopropyl-β-D-thiogalacto-pyranoside-induced protein band (~25 kDa) within a slice of the gel was used as an immunogen in rabbits (performed by Pocono Rabbit Farms and Laboratory, Canadensis, PA). We refer to this expressed recombinant human (rh) protein, which contains both the His6 tag and the signal peptide, as rh-pre-AMP-18. Preimmune sera were collected from two rabbits before injection of the protein in gel fragments. The ability of subsequent serum samples to react specifically with expressed rh-pre-AMP-18 was detected by immunoblotting, as described below. Specific antibodies were detected in both rabbits 2 wk after injection; titers reached a maximum after 4 wk.

Preparation and fractionation of antrum mucosal tissues. GI mucosal tissue from various sources was used in these studies. Pig antral mucosa from freshly slaughtered animals was cut from the lower layers of the tissue with scissors, rinsed with PBS, pH 7.4, and then homogenized on ice in TKM buffer (0.025 M Tris·HCl, 0.05 M KCl, 0.007 M MgCl2)
containing a cocktail of protease inhibitors (Roche Complete Cocktail; Roche Molecular Biologicals). Mucosal surface cells from murine (C57BL/6 strain, Taconic Farms, Germantown, NY) tissues were scraped into a Dounce homogenizer tube by using a razor blade or glass slide and were then homogenized on ice in TKM buffer with aprotonin (1 mg/ml). The homogenate was subjected to microcentrifugation for 30 s to remove debris, and the supernatant that resulted was then centrifuged for 30 min at 12,000 rpm. That second supernatant (referred to as the S2 fraction) contained AMP-18 was confirmed by immunoblot analysis. The S2 fraction was dialyzed overnight at 4°C against TKM buffer by using cellulose membrane dialysis tubing (Sigma D-9277) to remove proteins and peptides with a molecular mass smaller than 12 kDa. Then the dialysate was centrifuged to remove precipitated proteins; the supernatant was used for further study. Aliquots of the dialyzed extract were analyzed immediately in biological assays, fractionated further, or were stored frozen at −20°C. The biological activity and integrity of the 18-kDa antigen were stable for months under these conditions.

The antral mucosal supernatant fraction, as well as supernatant fractions prepared analogously from other tissues in the GI tract, were put into denaturing gel buffer and subjected to electrophoresis on a SDS-polyacrylamide (12.5%) gel. The separated proteins were transferred to nitrocellulose by conventional protocols. The blots were incubated with the antibody solution diluted 1:50,000 for immunoblot analysis. All procedures involving the use of animals were approved by, and in accordance with, the guidelines of the University of Chicago Animal Care and Use Resources Committee. Localization of AMP-18 protein. Formaldehyde-fixed, paraffin-embedded mouse antrum sections were reacted with rabbit preimmune (1:100 dilution) or anti-human pre-AMP-18 protein sera (1:1,000 dilution). Bound rabbit antibodies were then detected by using a peroxidase-labeled anti-rabbit antibody.

The localization of the AMP-18 antigens within mouse antrum mucosal cells was determined at the electron microscope (EM) level by a modification of our laboratory’s previous immunogold procedures (9). For these studies, the tissue pieces were fixed in 4% formaldehyde and processed for embedding in unicyril (Ted Pella, Redding, CA). Thin sections were reacted with rabbit anti-human pre-AMP-18 antisera (1:200); bound antibodies were detected by protein A conjugated to 10-nm colloidal gold (Pella). The reacted sections were stained with lead citrate before viewing. The preimmune sera (1:100) gave negligible background stain. Standard glutaraldehyde-osmium-epoxy sections of mouse antrum were also prepared for comparison.

Assays to detect growth-promoting activity of AMP-18 protein. The mitogenic activity of two different forms of AMP-18 was assessed: native AMP-18 in partially fractionated pig and mouse antral extracts, and rhAMP-18 produced in transformed E. coli, as described below.

Antrum extracts. To assess mitogenicity of the murine or porcine antral extracts, an aliquot was thawed and mixed with a solution containing an EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals). In some experiments, sections from SDS electrophoretic gels of the pig S2 fraction were extracted, and the renatured extracts were assayed for mitogenic activity. This was accomplished by cutting the gel into 2-mm slices that were extracted overnight at room temperature with 3% acetonitrile in PBS containing 1% BSA. An aliquot of the supernatant from each extract was then assayed for mitogenic activity in cultures of epithelial cells. The rabbit antiserum to rh-pre-AMP-18 blocked the mitogenic effect of the mouse and pig S2 fractions in all cases studied.

Preparation of rhAMP-18 protein. rhAMP-18, lacking the presumed signal peptide sequence, was prepared by cloning AMP-18 cDNA into a vector that expressed the AMP-18 ORF with an NH2-terminal sequence containing a His6 tag, but without the initial hydrophobic 20-amino acid peptide sequence encoded by the original full-length cDNA clone. The truncated AMP-18 cDNA construct was generated by PCR and cloned into the BamH1 site of a QE30 vector (Qiagen); recombinant vector clones were selected and sequenced. In-frame ligation was confirmed by sequencing. The correct recombinant vectors were transformed into BL21 (DE3)pLys5 E. coli cells to express rhAMP-18, a 177-amino acid protein containing the His6 tag (predicted M; 19,653). To harvest the protein, bacteria were lysed, and aliquots of the soluble and insoluble fractions were subjected to SDS-PAGE followed by immunoblotting using the specific rabbit antiserum to human pre-AMP-18. Very little of the expressed protein was detected in the soluble fraction of the lysate. Urea (6 M) was then employed to solubilize rhAMP-18 containing the His6 tag within inclusion bodies to make it available for binding to the Ni2+-charged resin from which it was subsequently eluted stepwise with imidazole (0–200 mM). The amount of eluted rhAMP-18 was measured by using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL), and the appearance of a single band at the predicted size of 19–20 kDa was confirmed by SDS-PAGE followed by immunoblotting.

Mitogenesis assays. Initially, mitogenic activity of antral extracts and solubilized rhAMP-18 was sought in epithelial-derived tissue culture cells of renal origin (nontransformed monkey BSC-1 line) that served previously to identify putative peptide growth factors (13, 26); we do not believe that kidney cells are the target of AMP-18 in vivo. This experimental strategy was also employed because a nontransformed gastric epithelial cell line is not available.

To measure DNA synthesis, high-density, quiescent cultures of BSC-1 cells were prepared by plating 2 × 10⁶ cells in 60-mm plastic dishes, as described previously (10, 24). Cells were grown in DMEM containing 1% calf serum and 1.6 μM biotin. The spent medium was changed after 3 days, and cultures were grown to a density of 3–4 × 10⁶ cells/dish. Medium was then aspirated and replaced with fresh medium containing 16 μM biotin and 0.01% calf serum; the quiescent cultures were used for study after 3 days. A specified amount of murine antrum cell extract protein in TKM was added to the medium of at least three cultures, and, 20 h afterwards, 12.5 μCi of [methyl-3H]thymidine (Amersham, 2 μCi/mmol) were added to each plate. Five hours later, radioactivity in the trichloroacetic acid-insoluble fraction was measured.

Proliferation of nontransformed BSC-1 or rat intestinal epithelial (IEC)-18 cells was measured by performing cell counts 3–4 days after exposing a confluent culture (10⁶ cells/60-mm dish) to the agent of interest. Trypsin was added to prepare a suspension of single cells that were counted in an hemocytometer, as reported previously (10, 13, 24, 26).

Statistics. Data were compared by Student’s t-test; P < 0.05 was accepted as significant. Values are means ± SE.

Reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified.

RESULTS

Isolation of antrum-specific cDNA clones. The cDNA clones that constituted the starting point for this project were side products of differential screening of a cDNA library prepared from pig stomach antral muco-
sal poly(A)-containing RNA obtained during isolation of the gastrin gene (28). A similar cDNA clone was subsequently isolated from a human antrum-specific cDNA library (17), and an equivalent mouse coding sequence was obtained by a combination of mouse genomic cloning (see below) and RT-PCR of mouse antrum RNA. The human, pig, and mouse coding sequences are compared in Fig. 1. Because these sequences code for a polypeptide present in antral mucosal cells that is an 18-kDa species, as shown below, we use the term AMP-18 to describe this gene product.

**AMP-18 gene structure and chromosome location in humans and mice.** We have used our human cDNA clones and mouse ESTs in GenBank to clone and sequence the following:

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<th>Species</th>
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<tr>
<td>Human</td>
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<tr>
<td>Pig</td>
<td>PIG000124</td>
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<td>Mouse</td>
<td>MUS000125</td>
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**Fig. 1. Alignment of human, pig, and mouse cDNA coding sequences for antral mucosal protein (AMP)-18.** Human and pig sequences were determined from full-length cDNA clones. The mouse sequence was determined from mouse genomic sequences confirmed by RT-PCR and RACE analysis of mouse antrum RNA. The ATG start site is at nucleotides nos. 44–46 in the human and nos. 40–42 in pig and mouse; the TAA/G termination site is at nucleotides nos. 600–602 in human, nos. 596–598 in pig, and nos. 593–595 in mouse. Exon-exon junctions are indicated by bold underlined capital letters, whereas poly(A) signals are italicized. A hyphen indicates a space between nucleotides, and a period denotes identical nucleotides between human and pig and/or mouse sequences. No. of nucleotides in the sequence is given in parentheses.
sequent the respective regions containing the AMP-18 gene. The 7,995-bp human and 7,280-bp mouse sequences have been submitted to GenBank (AY139182 and AY139183). Transcription start sites were determined by the RACE procedure. The AMP-18 transcription units in humans and mice have identical general plans of exons and introns, with the most apparent difference being the somewhat larger introns in the human gene (shown schematically in Fig. 2).

To map the mouse gene to its chromosomal location, we identified RsaI restriction polymorphism in a 253-bp fragment comprising exon 4/intron 4 junction from strain C57BL/6 and a wild mouse strain, M. spretus. This polymorphism was used in typing the 188 progeny of the Jackson Laboratory Mouse Backcross Mapping Panels. The results show that the gene is located in chromosome 6, 36 cM between the marker D6MIT8 (1 crossover in 188 progeny) and the gene Gata2 (3 crossovers in 94 progeny). From the mouse and human homology map (www.informatics.jax.org/reports/homologymap/mouse human.shtml), it is apparent that the human gene is very likely located on chromosome 2 at 2p13.

Tissue expression of AMP-18 mRNA. The original isolation of the cDNA clones was predicated on their preferential expression in the mucosa of the stomach antrum. Confirmation of tissue-specific expression was sought first by Northern blot hybridization of RNAs from various pig tissues probed with the cDNA sequence and subsequently by protein detection (see below). Figure 3 shows gastric mucosal specificity of AMP-18 mRNA expression among several tissues of the porcine GI tract; the size of the RNA is consistent with the full-length cDNA clone. The highest mRNA expression is in the antrum mucosa, with variable amounts in the adjacent corpus mucosa (possibly due to imprecise tissue excision), and undetectable levels in fundus, esophagus, and duodenum. The nonmucosal tissue of the antrum and corpus contained little RNA reacting with the cDNA probe (not shown).

Structural features of AMP-18 protein in humans, pigs, and mice. The pig and human cDNA clones contained ORFs, potentially encoding polypeptides of 185 amino acids (Fig. 4); the ORFs shown are considered most likely on the basis of a favorable AUG initiation codon. We present below protein evidence that the ORFs encode the precursors of secreted 18-kDa proteins and are, therefore, the predicted sequences of the precursor, i.e., pre-AMP-18, proteins. The human, pig, and mouse ORFs predict polypeptides with similar general structures (Fig. 4). Comparison of the predicted amino acid sequences reveals a high degree of identity (75.3%) and total similarity (78.5%) in the human and pig pre-AMP-18 proteins, and a lesser but still considerable degree of identity (63.9%) and similarity (70.5%) between human and mouse proteins. When all three amino acid sequences are subjected to secondary structure predictions (www.embl-heidelberg.

Fig. 2. Structure of the human and mouse pre-AMP-18 genes. Human and mouse genomic DNA sequences were amplified by using PCR techniques employing oligonucleotide primers based on the previously determined sequence of the human cDNA clone. The maps are presented approximately to scale (no. of base pairs in the introns are shown above the bars). Exons are indicated E1–E6 and introns I1–I5. Mouse AMP-18 exon sequences are sufficiently similar to those of human and pig to allow the complete sequence of the mouse pre-AMP-18 gene to be assembled, with slight uncertainty as to the exact site of transcription initiation. With the exception of minor differences in intron length, the structures of the genes in the 2 species are almost identical.

Fig. 3. Northern blot hybridization of RNAs from pig gut mucosal tissues. Total RNA was electrophoresed, transferred to a membrane, and hybridized with a 32P-labeled pig AMP-18 cDNA probe. The source of the RNA sample for each lane was as follows: 1, distal duodenum; 2, proximal duodenum; 3, antrum; 4, adjacent corpus; 5, fundus; 6, esophagus. Equal amounts of RNA were loaded. The signal from RNA in the antrum-adjacent corpus was variable; this example shows the highest level detected. The size markers (nucleotides) on the right were run on the same gel.
de/predictprotein/predictprotein.html), two consensus domains (A and B) emerge in the central region of the proteins, despite some differences in sequence among human, pig, and mouse. Domain B is predicted as a helix-loop-sheet, and our subsequent studies in the companion report (25) indicate an apparent biological role for this domain in AMP-18.

**Tissue expression of AMP-18 protein.** To study the biological role of AMP-18, the full-length cDNA sequence encoding the ORF of the human cDNA clone was expressed in *E. coli* by using a vector that also encoded an NH2-terminal His6 tag. Although the specifically induced polypeptide product (rh-pre-AMP-18) was insoluble in the absence of SDS, gel electrophoresis in the presence of SDS revealed a major ∼25-kDa band. This band was cut from gels and used as immunogen in rabbits. Two high-titer, low-background antisera were obtained that recognized both the immunogen and a protein ∼18–20 kDa in the antrum (at up to 1:50,000 dilution) of all seven different mammals (human, pig, mouse, dog, goat, sheep, rat) studied by immunoblotting; examples are shown in Fig. 5. This cross-reactivity of the antisera extends the level of conservation of amino acid sequence predicted by comparison of the ORFs of the human, pig, and mouse cDNAs (Fig. 1) to other species. When the antisera were reacted with diverse murine tissues, immunoreactive AMP-18 was detected in stomach antrum ex-
tricts and, to a much lesser degree, in adjacent corpus extracts, but not in duodenum, intestine, liver, brain, or kidney (Fig. 5). Nor was the protein found in mouse large intestine (cecum, transverse colon, rectum), spleen, pancreas, serum, gastric (AGS, HAE, KATO-III), or colonic carcinoma cell lines (CaCo-2, HT29) (not shown). The bacterially expressed rh-pre-AMP-18 protein migrates more slowly than native AMP-18 (Fig. 5), because the recombinant protein contains both a signal peptide sequence (see below) and an NH2-terminal His6 tag, a combination that appears to render it very insoluble. The preimmune sera give insignificant reactions on immunoblots of all tissue extracts. As the antisera were raised against a bacterially expressed protein, there is no possibility of other exogenous immunogens of animal origin.

**AMP-18 signal peptide.** Computer analysis of the amino acid sequences of human, pig, and mouse AMP-18 (Fig. 4) suggests the presence of a 20-amino acid signal peptide sequence at the NH2 termini whose cleavage is predicted to yield proteins of 165 amino acids (presumably 164 in mouse) after translocation across the endoplasmic reticulum membrane. The predicted molecular masses for the otherwise unmodified human and pig proteins would then be 18.3 and 18.0 kDa, respectively; these values are in good agreement with the electrophoretic mobility observed during SDS-PAGE of antrum proteins that react with the antisera (Fig. 5). By using immunoblots to fractionate the results of the immunoblots.

Localization of AMP-18 in cells of the gastric antrum. The original cloning strategy and subsequent nucleic acid and protein expression data implied that AMP-18 was specifically synthesized in antrum mucosal tissue, leading us to perform immunohistochemical studies to obtain more precise cellular location of the protein. The antisera are excellent immunohistochemical probes that react strongly with sections of mouse antrum (Fig. 6, B and C), but not with esophagus, duodenum, or intestine, confirming the results of the immunoblots. The preimmune sera give a negligible reaction (Fig. 6A), even at much higher concentrations. Immunoreactive AMP-18 protein appears to be concentrated in the mucosal epithelium lining the stomach lumen, although lesser signals in cells along the upper crypt regions suggest that cells may begin to express the protein as they migrate toward the luminal layer. Higher magnification reveals only general cytoplasmic staining at this level of resolution; there are some patches of intense staining that may be the light microscope equivalent of granule-packed regions of some luminal surface cells seen by EM (Fig. 7B). The localization of AMP-18 in the antrum mucosa is, therefore, very different from those cells synthesizing gastrin, which are deep in the mucosal layer (12).

Immunohistochemistry was also employed to study AMP-18 expression in cells of normal human gastric antrum and duodenum. All surface foveolar epithelial cells of the antrum, but only a few mucous cells of the...
contiguous duodenum, express AMP-18 (Fig. 6D). The protein was also detected, albeit to a far lesser extent, in the surface epithelia of the gastric body and fundus. AMP-18 was not found in the antral glands of the stomach, Brunner’s glands of the duodenum, lung, pancreas, or in the colon or rectum of normal humans or subjects with inflammatory bowel disease. AMP-18 was not detectable in either of two metastatic gastric cancers, or in gastric-appearing cells in Barrett’s esophagus.

A precise intracellular localization of AMP-18 was sought by using EM immunocytochemical procedures. Standard embedding and staining methods reveal that, as previously reported by others, the mouse antrum region contains mucosal epithelial cells that are very rich in mucin-containing secretory granules (7, 8). A very dense region toward the luminal surface of one cell is seen in Fig. 7B to contain granules of varying electron density. Immun-EM using sera at 1:200 to 1:800 dilution revealed a specific reaction with the secretion granules (Fig. 7A). The latter appear somewhat swollen and less electron opaque than in standard fixation conditions, and the differences in density are harder to discern, but, overall, the cell structure is quite well preserved for stomach tissue fixed and embedded under the less stringent conditions required to preserve immunoreactivity. Preimmune sera (1:100 dilution) exhibited negligible background signal with no preference for the secretion granules.

Growth-promoting activity of AMP-18. The acidic pH, mechanical stress, and active enzymes that facilitate digestion of food in the stomach lumen are possibly injurious for its mucosal lining cells. Therefore, we tested the hypothesis that AMP-18 is a growth factor at least partly responsible for maintaining a functional gastric epithelium by seeking evidence of epithelial cell mitogenic activity.

As AMP-18 is expressed primarily in the gastric antrum mucosa, we first set out to determine whether fractions prepared from antrum mucosal cell extracts were mitogenic. When immunoblotting was used to follow fractionation of lysed mucosal cells obtained from pig or mouse antrum, AMP-18 antigen was localized to the 3–5S region after centrifugation on sucrose density gradients. These high-speed supernatants (S2 fraction) were used to determine whether antrum extracts exhibited mitogenic activity. Initially, aliquots of mouse S2 fraction were assayed for their capacity to initiate DNA synthesis in high-density, quiescent cultures of nontransformed monkey BSC-1 epithelial cells. [3H]thymidine incorporation in control cultures increased from 4,942 ± 223 to 8,157 ± 498 cpm/culture (P = 0.003) when 2.5 μg/ml of S2 protein were added to the medium, to 11,463 ± 1,009 cpm/culture (P = 0.004) when 10 μg S2 protein/ml were added, and to 17,156 ± 1,069 cpm/culture with 20 μg S2 protein/ml (P < 0.001). Stimulation of [3H]thymidine incorporation by antrum extract was totally inhibited by the addition of the specific rabbit antiserum to human pre-AMP-18 (1:100 dilution), but was unaffected by the presence of preimmune serum (1:100 dilution) (data not shown). This result suggested that the active mitogen in antrum extract is native AMP-18. We then asked if stimulation of [3H]thymidine incorporation was correlated with increased cell proliferation, as has been observed previously with other mitogenic signals in BSC-1 cells (10, 24). When added to confluent cultures, the S2 fraction (10 μg protein/ml) increased the number of cells by 275% during 4 days in culture, compared with a 180% increase in cultures to which only vehicle was added (P < 0.001). Cell proliferation was up to twice that observed with diverse polypeptide mitogens (EGF, IGF-I, aFGF, bFGF, vasopressin) assayed at their maximal concentrations (data not shown). As these cells are observed not to clump after trypsinization, an

Fig. 7. Immuno-electron microscope localization of AMP-18 antigens in mouse stomach antrum mucosal cells. The tissue pieces were fixed in formaldehyde (4%) and processed for embedding in Unicryl. B: electron micrograph of a single surface epithelial cell showing granules in subapical region (×11,400). A: thin sections were reacted with the specific rabbit antiserum to AMP-18 (1:200); bound antibodies were detected by protein A conjugated to 10 nm colloidal gold. Reacted sections were stained with lead citrate before viewing (×20,000). Gold particles are visible over the semitranslucent secretion granules, which appear much more translucent here than in the standard glutaraldehyde-osmium-epoxy procedure (used to visualize cell shown in B) because of the requirements for immunoreactivity. Negligible background is seen on other cytoplasmic structures.
increase in cell number provides direct evidence of growth-promoting activity (10, 13, 24, 26).

We also found that the mitogenic activity of antrum extracts survived heating for 5 min at 65°C and subsequent dialysis with the use of a membrane with a molecular mass cutoff of 10 kDa to eliminate most oligopeptides; this treatment removed 60–70% of peptide material, but spared AMP-18 as assayed by immunoblots. More importantly, mitogenic stimulation of the epithelial cells by pig or mouse antrum extract was inhibited ($P < 0.001$) when either antiserum to pre-AMP-18 was added to the culture medium (Fig. 8). Preimmune sera (1:100 to 1:800) had no effect on cell growth, nor did they alter the mitogenic effect of the antrum extracts. These observations suggested that AMP-18 is an epithelial cell mitogen.

To obtain additional evidence that the growth-promoting activity in partially fractionated antrum extracts was mediated by native AMP-18, an aliquot of porcine extract was subjected to SDS-PAGE. The gel was cut into 2-mm slices, which were extracted, and an aliquot of the supernatant from each extract was then assayed for mitogenic activity. The results indicated that one slice containing a ∼18-kDa protein possessed growth-promoting activity (Fig. 9) that was blocked by the immune, but not the preimmune, sera. Taken together with the relatively low-sedimentation rate of the protein (3–5S), these findings provided additional evidence to support our conclusion that AMP-18 is an epithelial cell mitogen that functions as a monomer or possibly a homotypic dimer. The results imply that the structure of the protein is such that it can reacquire a native conformation after experiencing the denaturing conditions of SDS-PAGE.

**rhAMP-18 prepared in E. coli is an active mitogen.** A cDNA encoding human AMP-18 was designed in which the 20-amino acid hydrophobic signal peptide sequence was replaced with an NH$_2$-terminal, 12-amino acid peptide that included a stretch of six histidine residues (His6 tag). Expression of this modified cDNA sequence was predicted to yield a 177-amino acid protein product ($M_r$: 19,653) that was readily purified by using a nickel-affinity column to bind the His6 tag. After its elution from the Ni-NTA resin, rhAMP-18 apparently renatured to assume a structure that was mitogenic, because aliquots of the eluate (following removal of urea and imidazole by dialysis) stimulated proliferation of nontransformed rat IEC-18 cells at a half-maximal

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**Fig. 8.** Growth stimulation of epithelial cells by murine (A) or porcine (B) antrum extract is blocked by the specific rabbit antiserum to human pre-AMP-18. Murine or porcine antrum cell extract was prepared, and its protein concentration was measured. Cell extract protein, alone and with different dilutions of the antiserum, or antiserum alone (1:100 dilution) was added to the medium of confluent BSC-1 cell cultures. The no. of cells was counted 4 days later. Growth stimulation by murine antrum extract was inhibited maximally (93%) by the antiserum at a dilution of 1:400 ($P < 0.001$). Stimulation of cell growth by porcine antrum protein extract was totally inhibited at a dilution of 1:100 ($P < 0.001$) and, to a lesser extent, at dilutions of 1:200 and 1:400 ($P < 0.001$). At a dilution of 1:800, the antiserum did not inhibit the mitogenic effect of either murine or porcine antral extracts ($P =$ not significant). Values are means for 3 cultures; SE is <10% of the mean.
protein concentration of 5 nM (Fig. 10). The mitogenic effect of rhAMP-18 was blocked by the antiserum to pre-AMP-18. That the mitogenic potency of rhAMP-18 is in the nanomolar range suggests that this growth factor protein could participate in the maintenance and repair of the stomach in vivo.

**DISCUSSION**

AMP-18 is a novel protein expressed primarily in surface epithelial cells of the gastric antrum and is apparently stored in mucin-containing secretion granules. The human and mouse genes have identical genomic structural features, and chromosome mapping indicates that AMP-18 is located on chromosome 6 in the mouse and chromosome 2 in humans (consonant with our detection of the protein in sheep abomasum mucosal extracts. The tissue-specific expression of AMP-18 protein is of some interest because our analysis of both RNA and protein suggest a high degree of specificity to the mucosa of the gastric antrum. Related ESTs have been reported in several databases, suggesting much lower levels of expression in a number of other tissues, including pancreas; some human and mouse EST data are presented at www.tigr.org/tdb/hg/ and www.tigr.org/tdb/mgi/. microarray data for mouse ESTs are reported at read.gsc.riken.go.jp (11).

There is one major discrepancy between the mouse EST microarray data and our protein determinations: whereas the former report high expression in the cecum, we have been unable to detect the protein in immunoblots prepared from the cecal mucosa of multiple mice. However, it would be premature to disregard the relatively low levels of expression of AMP-18 in non-GI tissues cited in the reported ESTs, given the physiological implications of the mitogenic potency of AMP-18 on cultured epithelial cells.

Recently, Yoshikawa et al. (30) and Shiozaki et al. (21) reported a cDNA that predicts an amino acid sequence that differs from AMP-18 in only a single amino acid. The clone was identified in normal but not in cancerous human gastric tissue by using a differential display technique. Called CA11, the cDNA was not found in human gastric cancer cell lines, which confirms our experience to date. We do differ with these investigators as to the start site of AMP-18 precursor.

We have shown by immunoblotting that AMP-18 proteins exist in the stomachs of seven mammalian species; an homologous sequence has been detected in a bovine abomasum (ruminant stomach) [cDNA library (www.tigr.org/tdb/tgi/btgi/), identifier: TC101852], consistent with our detection of the protein in sheep abomasum mucosal extracts. The tissue-specific expression of AMP-18 protein is of some interest because our analysis of both RNA and protein suggest a high degree of specificity to the mucosa of the gastric antrum. Related ESTs have been reported in several databases, suggesting much lower levels of expression in a number of other tissues, including pancreas; some human and mouse EST data are presented at www.tigr.org/tdb/hg/ and www.tigr.org/tdb/mgi/. microarray data for mouse ESTs are reported at read.gsc.riken.go.jp (11).
They put it 14 amino acids upstream from the start site shown in Fig. 1. Our designation of the translation start site is based on initiation sequence prediction and the actual sequence of the NH2 terminus of native AMP-18 obtained from purified porcine antrum extract eluted from an SDS-PAGE gel, which would result from the in vivo processing of a perfect 20-amino acid signal peptide; the addition of 14 amino acids upstream would abolish the predicted signal peptide. The one amino acid difference between the sequences is an aspartate at AMP-18 position no. 25 (Fig. 1), whereas an asparagine is reported in that position in CA11. We cannot say whether there is a sequencing error, or whether there are polymorphisms at this locus. Finally, Yoshikawa et al. (30) and Shiozaki et al. (21) have not assigned a function to their putative protein. We have observed that AMP-18 has mitogenic activity, and this may be seen as a contradiction to a possible antitumorigenesis role implied by these authors, as neither they nor we detected the protein in gastric cancer tissue (21, 30). However, the progressive synthesis of AMP-18 in gastric epithelial cells as they climb the walls of the crypts toward the mucosal surface suggests that the protein is expressed postmitotically and could, in addition, function in an epithelial maturation role, a possibility tested in studies described in the companion report (25).

Immunoelectron microscopy suggests that AMP-18 is cosecreted with gastric mucin. If so, it could serve as a normal constituent of the mucus overlying the antrum epithelium in vivo. As a component of the mucin layer, AMP-18 could provide mechanical and chemical protection for the underlying mucosa, by acting as a lubricant or gel stabilizer during digestion, defending surface mucosal cells against stomach acid, and perhaps helping to maintain the mucous pH gradient and/or hydrophobic barrier. The trefoil peptide family has been suggested to have such general cytoprotectant roles (20) and may act in concert with AMP-18 and/or hydrophobic barrier. The trefoil peptide family also has been suggested to have such general cytoprotectant roles (20) and may act in concert with AMP-18 and other factors to maintain and repair the GI epithelium (16). Our results suggest that AMP-18 in the antrum, either directly or indirectly, as a gastric epithelial growth factor. The GI tract represents the largest surface area for interaction between the host and microorganisms, and a number of antimicrobial peptides has been described for the intestine. The gastric antrum region is the favored site for the attachment, penetration, and cytolytic effects of Helicobacter pylori, an agent that infects a major proportion of the human population (>60% by the seventh decade of life) and has been associated with gastritis, gastric and duodenal ulcers (1, 5), and gastric adenocarcinomas (14, 15). Thus AMP-18 released into the gel overlying the mucosal surface might speed recovery after epithelial damage caused by diverse pharmacological agents, as well as bacterial infiltration and cytolyis.

Because the stomach mucosa is in constant need of regeneration because it is subject to harsh conditions (mechanically and chemically), it may not be surprising to find that the cellular cells of the antrum secrete a growth factor that could play an autocrine and/or paracrine role. Our results support the hypothesis that AMP-18 is secreted at, and could act on, the apical face (i.e., stomach lumenal face) of the epithelial cell layer, whereas other factors, such as EGF, may act from the basolateral surface. This would be similar to the role of the trefoil peptides, suggested to act from the “outside” in collaboration with other factors, such as cytokines acting from the “inside” to maintain or reconstitute an intact functional epithelium (16). Whereas AMP-18 shares no immediately obvious structural similarities with the trefoil peptides and has a different site of synthesis within the mucosal epithelium (20, 27), its mitogenic potential suggests a role in the growth-regulatory physiology of the stomach (22). An alternative hypothesis is that, after mucosal injury, secreted AMP-18 protein or its peptides could reach and act at the basolateral epithelial cell surface as a consequence of disrupted tight junctions and diminished barrier function.

Our RNA and protein blots, coupled with the immunocytochemistry and the ability to isolate the protein by gel electrophoresis from crude extracts, suggest a substantial level of AMP-18 expression in the gastric antrum mucosa. Furthermore, the relative frequency of AMP-related ESTs reported in the public databases imply that AMP-18 RNA may represent 1–5% of total antral mucosal cell mRNA. This raises the question of why there is a need for substantial expression of a protein that is a putative growth factor and that has considerable potency when assayed in culture. One plausible explanation is suggested by the extreme physical and chemical environment of the gastric lumen, which may be expected to rapidly turn over secreted protein factors. It may also be that AMP-18 has additional physiological activities, perhaps as derived peptides. Further evidence that AMP-18 is secreted by gastric antral mucosal cells and has mitogenic and/ or reparative properties that could permit it to maintain and repair the stomach epithelium are presented in the report that follows (25).

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