Peptide fragments of AMP-18, a novel secreted gastric antrum mucosal protein, are mitogenic and motogenic

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Cells of the gastric mucosa are constantly subjected to the stresses of an acidic pH, the proteolytic enzyme pepsin, and high pressures that develop in the stomach lumen during digestion. Identification of endogenous molecules that protect the mucosa in this hostile environment and facilitate repair of surface epithelial cells when they are injured would provide fresh insight into stomach physiology. A cDNA clone encoding a protein with these characteristics was identified, but not recognized as such, during the cloning of the peptide hormone gastrin, more than a quarter century ago. During the original isolation of cDNA clones that encode gastrin, which is expressed specifically in cells of the gastric antrum (31), another antral-specific mRNA was identified (21). Its open-reading frame, which is highly conserved between human and pig, predicted a novel conserved protein of no readily predicted function (11). The cDNA was expressed in E. coli, and the protein product was used to prepare two specific polyclonal antisera in rabbits. The antisera were subsequently used to localize the protein in the antral mucosa of all seven mammals tested to date (11). Given the tissue specificity of expression of the cDNA sequence and the apparent ubiquitous presence of the protein in the antrum mucosa of mammalian species, the molecule was named AMP-18 for 18-kDa antrum mucosal protein. A cDNA clone called CA11, which predicts an amino acid sequence that differs from human AMP-18 in only a single residue (11), was reported in normal human gastric mucosa, but not in most gastric cancers, by using a differential display technique (25, 32). Although a function for CA11 protein was not identified by the investigators who discovered it, they did suggest that loss of CA11 expression may play a role in gastric carcinogenesis.

Expression of AMP-18 was localized to mouse gastric antrum by using immunohistochemistry and immunoblotting and by Northern blot hybridization of RNAs from porcine gut mucosal tissues (11). Immunelectron microscopy indicated that the protein is localized within granules just under the apical plasma membrane, suggesting that it is a secreted rather than an integral membrane protein. Initial studies to identify a function of the protein showed that porcine and murine antrum extracts were mitogenic for epithelial cells in culture, and that this growth-promoting effect was blocked by each of two specific antisera (11). A recombinant human (rh) protein was also found to be mitogenic. These observations stimulated us to better characterize release of AMP-18, seek a mitogenic domain within its primary structure, and determine whether it was capable of restitution to learn more about how it could maintain and/or repair the gastric epithelium.

MATERIALS AND METHODS

SDS-PAGE and immunoblotting. Proteins in canine gastric antral mucus, the medium of primary cultures of canine antral cells, and mouse antral tissue (obtained as described...
below) were homogenized in lysis buffer (10 mM Tris, pH 7.4, 5 mM MgCl₂, 50 mM U/mL DNase and RNase, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin, apro tinin, and pepstatin), solubilized in a half-volume of 3% Laemmli sample buffer containing β-mercaptoethanol, separated by SDS-PAGE (12.5%), and blotted onto a polyvinyldene difluoride (Immobilon P; Millipore, New Bedford, MA) membrane by using Towbin buffer (25 mM Tris, 122 mM glycine, 20% methanol, pH 8.3). Nonspecific binding sites were blocked by using 5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS; 100 mM NaCl, 10 mM KCl, pH 7.3) with 0.05% (vol/vol) Tween 20 (T-TBS) for 1 h at room temperature, as described previously (1). The membrane was then incubated with primary antibody [rabbit antiserum to rh AMP-18 precursor (rh-pre-AMP-18)], described previously (11), or mouse monoclonal anti-cytokeratin peptide 18 clone CY-90 diluted in T-TBS. It was rocked gently overnight at 4°C and then washed four times in T-TBS at room temperature for a total of 30 min. The immunoblot was incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h and washed three times with T-TBS. The T-TBS; the labeled proteins were visualized with an enhanced chemiluminescent reagent (SuperSignal West Pico, Pierce, West Rockford, IL).

**Release of AMP-18.** Lysates of confluent cultures of different gastrointestinal (GI) epithelial cell lines were prepared to study the release of AMP-18. Ten micrograms of total cell protein were assayed for the presence of AMP-18 by immunoblotting lysates prepared from several established gastric lines [AGS, human antral epithelial (HAE), KATO III, RGM-1, NCI-N87, SK-GT3], a gastric line derived from the Immortomouse (30) (gift of R. H. Whitehead, Vanderbilt University) at the permissive or nonpermissive temperature, human colonic adenocarcinoma lines (HT29A1 and CaCo2/bbe, subclone C2), and nontransformed monkey and canine epithelial cells (IEC) of the IEC-6 and IEC-18 lines, NCI-N-87 human gastric carcinoma cells, SK-GT5 human gastroesophageal adenocarcinoma cells, and monkey kidney epithelial BSC-1, Madin-Darby canine kidney) epithelial cells, none of which expressed the protein. As the protein was not detected in tissue samples from primary or metastatic human gastric carcinomas by immunohistochemical staining, and a nontransformed human gastric epithelial cell line does not exist, preliminary studies were performed by using mouse gastric antrum explants and, subsequently, in primary canine antrum cell cultures. AMP-18 in the medium bathing antrum explants was concentrated with a YM-10 filter and once with TBS; the labeled proteins were visualized with an enhanced chemiluminescent reagent (SuperSignal West Pico, Pierce, West Rockford, IL). To measure mitogenic activity, AGS human gastric adenocarcinoma cells, HAE (human gastric antral epithelial primary cultures transformed with SV40 large T antigen; kindly provided by Dr. Duane Smoot, Howard University College of Medicine), rat diploid small intestinal epithelial cells (IEC) of the IEC-6 and IEC-18 lines, NCI-N-87 human gastric carcinoma cells, SK-GT5 human gastrointestinal adenocarcinoma cells, and monkey kidney epithelial BSC-1 cells (11) were studied. Human WI-38 fibroblasts and HeLa cells served as non-GI control cell lines. Mitogenesis was assayed by performing cell counts 4 days after exposing a confluent culture to the agent of interest, adding trypsin to prepare a suspension of single cells, and confirming cell separation while counting them in a hemocytometer, as reported previously (28). To measure DNA synthesis, IEC-6 cells were plated at a density of 3 × 10⁵ cells per 80-mm dish and grown to high density in DMEM containing 1% calf serum (CS) and insulin (100 μU). The medium was replaced with fresh medium containing 0.01% CS and insulin; cells became quiescent and were used for study 2 days later. The agent of interest (in water) or vehicle (water) was added to the culture medium, and, 20 h afterwards, 12.5 μCi of [methyl-³H]thymidine were added. Five hours later, radioactivity...
in the trichloroacetic acid-insoluble fraction was measured, as described previously (28).

Mitogenic activity was assessed in each of the following preparations: native AMP-18 in pig antral extracts (11), rhAMP-18 produced by transformed E. coli obtained as described previously (11), and synthetic peptides derived from a central domain of the predicted sequence of mature human AMP-18. Preparations were each purified by reverse-phase HPLC by using a gradient of acetonitrile (1–80%) in 0.09% trifluoroacetic acid. The sequence of each peptide was confirmed by microsequencing, and its predicted size was confirmed by mass spectrometry. The purified peptide was then dissolved in water, and its capacity to stimulate growth of cells in culture was assessed, as described above.

Restitution in scrape-wounded monolayer cultures. To measure migration after scrape wounding (5, 6, 18), HAEC or IEC-18 cells were grown to high density in a-50 culture medium (Biosource International, Rockville, MD) or DMEM, respectively, containing 1% CS, in 60-mm dishes. Each medium was aspirated and then replaced with fresh medium containing 0.01% CS. The monolayer was mechanically wounded by scraping off a section of it with a razor blade. Detached cells were removed by aspirating the medium and rinsing the remaining cells twice with fresh medium containing 0.01% CS. Fresh medium (5 ml) containing CS (0.01%) and insulin (100 U/l) was added to scrape-wounded cultures. Either a synthetic AMP peptide, EGF, or both were added to duplicate cultures. Migration was assessed at 24, 48, and 72 h after wounding by measuring the distance (in mm) that cells had migrated from the wound edge by using a microscope eyepiece reticle (10 mm long; 0.1-mm markings). The distance traveled by migrating cells at 12 randomly chosen sites along a 0.25-mm segment of the wound edge was measured at 40-fold magnification. Migration was assessed at different sites in two separate wounds made in each culture.

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

Secretion of AMP-18 by gastric mucosal epithelial cells. Evidence for secretion of AMP-18 in vivo was sought by aspirating aliquots of mucus overlying the gastric antrum of three fasted, anesthetized dogs without perturbing the mucosal surface. As shown in the right lane of Fig. 1, a representative immunoblot of this mucus revealed abundant AMP-18 with only a very weak signal for cytokeratin-18, a marker protein used to detect desquamated gastric epithelial cells. These results, taken together with the previous immunoelectron microscopic localization of AMP-18 in secretion granules (11), suggest that AMP-18 is released from the cells into the mucus that overlies the antral mucosa, rather than being deposited there when detached surface cells degenerate.

Next we asked if secretion of intracellular AMP-18 is subject to regulation. Although we were unable to identify an established gastric, colonic, or renal epithelial cell line that contained the protein, a pilot study using mouse antrum explants was employed to test the hypothesis that AMP-18 is released by the cells. Immunoblot analysis of proteins in the explant bathing medium revealed that AMP-18 was present in the serum-free, buffered salt solution at pH 7, as well as at pH 3, and, to a greater extent, at 37°C than at room temperature (data not shown). Next, primary cultures of canine antral epithelial cells were prepared (2) and shown to contain AMP-18 when immunoblotting was performed on extracts of cell monolayers. Forskolin, a compound known to raise intracellular cAMP, was added to the monolayer to determine whether this second messenger acts as a secretagogue for AMP-18 as it does for parathyroid hormone (3). Measurements based on immunoblots of cell lysates indicated that AMP-18 immunoreactivity declined by 38% (P < 0.05) 1 h after exposure to forskolin (Fig. 2).

To determine whether AMP-18 release is triggered in vivo by an agent known to act on the gastric antrum of humans and rodents (13, 24), indomethacin, a non-selective COX inhibitor, was gavaged into C57BL/6 mice. Immunoreactive AMP-18 in antral mucosal scrapings was reduced by 70% in animals given indomethacin compared with control animals at 4 h (P < 0.02) (Fig. 3). However, no histological evidence of gastric mucosal injury in the treated mice was detected before 18 h, as reported previously (13). In addition to the negative histological findings, further evidence that indomethacin did not induce cell detachment was obtained when immunoblots revealed no differences between control and indomethacin-treated tissue when probed with an antiserum to cytokeratin-18. As in the immunoblots (Fig. 3), immunohistochemical analysis of tissue from mice exposed to indomethacin for 8 h revealed less AMP-18 within cells of the antral surface and upper crypts than in control animals gavaged with the vehicle (not shown). The apparent absence of mucosal cell detachment by histological and immunohistochemical techniques suggests that exposure to a non-selective COX inhibitor decreases mucosa cell content.
lysine-78 to leucine-119 of the pre-AMP-18 sequence), exhibited mitogenic activity ($K_{1/2} = 0.3 \mu M$) (Table 1). Its growth-promoting activity was totally blocked by the specific antisera, but not the preimmune sera, and immunoblots showed that the antisera recognized epitope(s) on the synthetic peptide (not shown). The reaction of AMP peptide 58–99 with the antibodies was not unexpected, because this region of the sequence is predicted to be exposed on the surface of the protein and to be antigenic. Synthetic peptide 58–99 appears to exert its growth-promoting effect via the same pathway as native AMP-18, because their maximal mitogenic effects are not additive (not shown).

To more rigorously define the mitogenic domain, the sequence of the 42-mer (peptide 58–99) was divided so that a lysine-lysine (K-K) doublet was at the NH$_2$ terminus, and a single K was at the carboxy (C)-terminus of each of three new peptides that were synthesized, HPLC purified, and assayed for mitogenic activity (Table 1). Peptides 58–68 and 67–85 were inactive. Growth was stimulated by peptide 84–97, but required a higher molar concentration to reach a similar maximal value than did peptide 58–99; this is reflected in the higher $K_{1/2}$ (0.8 $\mu M$), which suggests that peptide 84–97 (a 14-mer) has only 38% of the activity of the 42-mer.

Peptide 84–97 was then extended in the NH$_2$-terminal or COOH-terminal direction to determine whether a slightly longer peptide would replicate the greater mitogenic potency of the 42-mer. Peptide 77–97 was synthesized by extending peptide 84–97 by seven amino acids toward the NH$_2$ terminus, whereas pep-
The small size of peptides 84–97 mer was not mitogenic at concentrations up to 120 µM, indicating that they were not as potent (Table 1). A K<sub>1/2</sub> of 0.3 µM for peptides 58–99 and 77–97 imply equivalent mitogenic potency, despite the twofold difference in their lengths. Amino acids 77–97 appear, therefore, to represent the mitogenic domain contained within the 42-mer (peptide 58–99). When peptide 77–97 was divided into smaller fragments, peptides that were 6, 9, 14, and 18 amino acids in length were each mitogenic, but their K<sub>1/2</sub> values were higher than for the 21-mer, indicating that they were not as potent (Table 1). A 4-mer was not mitogenic at concentrations up to 120 µM. The small size of peptides 84–101 (18-mer), 84–97 (14-mer), 89–97 (9-mer), and 84–89 (6-mer) suggest that they exert their mitogenic effects via a receptor-mediated mechanism, because none of them is long enough to extend through the plasma membrane, which usually requires a minimum of 20 amino acids. Peptide 77–97, a 21-mer, seems unlikely to insert into the plasma membrane, because its amino acid composition predicts a strongly hydrophilic character; its relative hydrophobicity value (-44.4 kcal/mol).

AMP-18 and its derived peptides stimulate growth of stomach and IECs. To assess the role of AMP-18 as a gastrin cell growth factor, its effect on proliferation of four human stomach lines (AGS, HAE, NCI N-87, SK-GT5) was studied. Mitogenic stimulation of AGS cells was observed with porcine antrum mucosal tissue extract and synthetic human AMP peptide 77–97 (Fig. 4, top). As expected, rabbit antiserum to AMP-18 precursor protein inhibited growth-promoting activity of the antrum extract, but not of the much smaller peptide 77–97, suggesting that the mitogenic 21-mer lacks the epitope(s). In AGS cells, growth stimulation by peptide 77–97 was additive with the maximal mitogenic concentration of EGF (P < 0.001), suggesting that the two mitogens do not use the same receptor and/or utilize different signaling pathways (Fig. 4, top right). The scrambled isoform of peptide 77–97 (Table 1) did not stimulate the growth of AGS cells (data not shown).

HAE cells were studied to test whether AMP-18 could exert its mitogenic effect on epithelial cells that exist in the local environment of its synthesis in vivo. Fig. 5, left, shows that AMP peptide 77–97 stimulated growth of these cells, as did EGF (P < 0.001). Growth of NCI N-87 cells and SK-GT5 cells was also stimulated by porcine or murine antrum extract, peptide 77–97, or EGF in a concentration-dependent manner (data not shown). Antiserum to AMP-18 blocked the mitogenic effect of antrum extract on these two gastric epithelial cell lines, but not the proliferative effects of peptide 77–97 or EGF. As each of the four human stomach epithelial cell lines studied is transformed, and a nontransformed gastric epithelial cell line is not available, we also studied nontransformed, epithelial lines from two other species: rat intestinal IEC-6 and IEC-18 cells, and monkey kidney BSC-1 cells. As with the gastric epithelial cells, growth of rat diploid IEC-6 cells was also stimulated by the antrum extract, peptide 77–97, and EGF, although the peptide appeared to be a more potent mitogen than EGF (Fig. 4, bottom right) (P < 0.001). The mitogenic effect of peptide 77–99 was corroborated by measuring [3H]thymidine incorporation into DNA in confluent cultures of IEC-6 cells, which was stimulated by 68% (P < 0.001), from 16,668 ± 616 counts per minute/10<sup>5</sup> cells in control cultures to 28,036 ± 882 counts per minute/10<sup>5</sup> cells exposed to AMP peptide (8 µg/ml) for 24 h. Preliminary sera had no effect on growth. Purified rhAMP-18 stimulated growth of IEC-18 cells to the same extent as did AMP peptide 77–97, but the K<sub>1/2</sub> required by the peptide (300 nM) (Table 1) was far greater than that for the recombinant protein (5 nM).

**Table 1. Analysis of the mitogenic domain of AMP-18**

<table>
<thead>
<tr>
<th>Peptide Name*</th>
<th>No. AA</th>
<th>Sequence</th>
<th>K&lt;sub&gt;1/2&lt;/sub&gt; µM</th>
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</thead>
<tbody>
<tr>
<td>58–99†</td>
<td>42</td>
<td>KKTCIVHKMKKEVMPISQLDALVKEKKLQGKPQGPPKGL</td>
<td>0.3</td>
</tr>
<tr>
<td>58–68</td>
<td>11</td>
<td>KKTCIVHKMKKK</td>
<td>1</td>
</tr>
<tr>
<td>67–85</td>
<td>19</td>
<td>KKEVMPISQLDALVEKKK</td>
<td>1</td>
</tr>
<tr>
<td>84–97</td>
<td>14</td>
<td>KKLCQGGPGPPK</td>
<td>0.8</td>
</tr>
<tr>
<td>84–101</td>
<td>18</td>
<td>KKLQGKGPGPPKGLMY</td>
<td>1.0</td>
</tr>
<tr>
<td>77–97</td>
<td>21</td>
<td>LDALVKEKKLQGKGPGPPPK</td>
<td>0.3</td>
</tr>
<tr>
<td>77–97 scrambled</td>
<td>21</td>
<td>GKPGGPPK</td>
<td>2.5</td>
</tr>
<tr>
<td>77–101</td>
<td>25</td>
<td>KKLQGK</td>
<td>7.4</td>
</tr>
<tr>
<td>89–97</td>
<td>9</td>
<td>KGLMY</td>
<td>1</td>
</tr>
<tr>
<td>84–89</td>
<td>6</td>
<td>GPG</td>
<td>0.2</td>
</tr>
<tr>
<td>90–93</td>
<td>4</td>
<td>VP</td>
<td>0.2</td>
</tr>
<tr>
<td>77–99 mouse</td>
<td>23</td>
<td>LDTMVKEQK–GKPGGAPPKDLMY</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Predicted sequences of all peptides are based upon human open-reading frame of 18-kDa antrum mucosal protein (AMP-18)(11), except for mouse peptide 77–99 (11). Peptides are identified by their position in the amino acid sequence of the mature form of AMP-18, i.e., after signal peptide region has been removed. Each space between amino acids is denoted by a hyphen (–), which aligns the human and mouse sequences. A critical 14-amino acid mitogenic domain is in bold type. AA, no. of amino acids in peptide; K<sub>1/2</sub>, concentration (µM) for half-maximal growth stimulation of BSC-1 cells; I, inactive. †This synthetic peptide was based on the original determination of the sequence by Powell and Agarwal (unpublished observation). Our reevaluation of the human sequence predicts asparagine at position 67 (11); the pig protein appears to have a lysine at this position.
Fig. 4. Effect of porcine antrum mucosal extract, human AMP peptide 77–97, and EGF on growth of gastric (AGS) and intestinal (IEC-6) cells. Different amounts of antrum extract, peptide 77–97, and/or EGF were added to the culture medium, and 4 days later the no. of cells was counted. Antrum extract and AMP peptide each stimulated growth of both AGS (top left and middle) and IEC-6 (bottom left and middle) cells (P < 0.001 at 2 μg/ml) in a concentration-dependent manner. Values are means ± SE for at least 4 cultures. When antrum extract (2 μg/ml) was preincubated for 30 min with rabbit antiserum to human AMP-18 (1:100 dilution; Ab) before addition to the culture medium, growth stimulation was reduced by 90% (top left, △) (P < 0.001); preimmune serum had no effect (data not shown). The antiserum did not alter the mitogenic effect of peptide 77–97 (2 μg/ml) (top middle, △). The mitogenic potency of peptide 77–97 (8 μg/ml) (P < 0.001) was similar to EGF (50 ng/ml) (P < 0.001) in AGS cells (top right), but the peptide (1 μg/ml) appeared more potent than EGF in IEC-6 cells (bottom right) (P < 0.001). Cont, control.

Fig. 5. Effect of AMP peptide 77–99 and EGF on growth and wound restitution of human antrum epithelial cells. To measure growth (left), human antrum epithelial (HAE) cells were plated in 60-mm dishes. Peptide 77–97 (8 μg/ml) or EGF (50 ng/ml) or both were added to the medium, and the no. of cells was counted 4 days later. AMP peptide and EGF each stimulated proliferation (P < 0.001). Values are means ± SE for at least 5 cultures. To measure migration (right), cells were grown in 60-mm dishes to obtain a confluent monolayer. The medium was aspirated and replaced with fresh medium containing 0.01% calf serum (CS), and the monolayer was mechanically wounded by scraping with a razor blade. Detached cells were removed by aspirating the medium and rinsing the wounded monolayer twice with fresh medium containing 0.01% CS, and the monolayer was mechanically wounded by scraping with a razor blade. Detached cells were removed by aspirating the medium and rinsing the wounded monolayer twice with fresh medium containing 0.01% CS. Fresh medium (5 ml) containing 0.01% CS alone or with either peptide 77–97 (8 μg/ml), EGF (50 ng/ml), or both was added to each of 2 wounded cultures. Migration was assessed at 72 h after wounding by measuring the distance (in mm) that cells had migrated from the wound edge, as described in MATERIALS AND METHODS. Values are the mean distance that cells migrated into the denuded area from the edge of 2 different wounds in each of 2 cultures ± SE. Cells exposed to peptide 77–97 migrated further from the wound edge than those exposed to vehicle (P < 0.001). EGF also stimulated wound resurfacing, and the 2 agents appeared to act in an additive manner to enhance migration (P < 0.001). Cont, control.
Competitive mitogenic inhibition by AMP-18-derived peptides. To better characterize the apparent interaction between AMP peptides and their binding site(s) on the cell surface, nontransformed rat IEC-18 cells were studied. We tested the hypothesis that progressively increasing the concentration of nonmitogenic peptide 67–85 would block growth stimulation by peptide 58–99 if this mitogenic 42-mer exerts its effect by a receptor-mediated mechanism. Peptide 58–99 stimulated an increase in cell number of 407% compared with 290% by the vehicle in a 3-day assay ($P < 0.001$). As the concentration of peptide 67–85 was raised progressively to $\sim 0.1 \mu g/ml$, the growth-stimulatory effect of peptide 58–99 was nearly abolished ($P < 0.001$) (Fig. 6), suggesting that the two peptides compete for the same surface “receptor” site.

Restitution after scrape wounding. As restitution is an important component of wound repair, and mitogenic proteins such as EGF are also motogenic (5), we added AMP peptide to scrape-wounded monolayer cultures of HAE cells and found that it stimulated migration of cells at the wound edge at 72 h (Fig. 5, right). This enhancement of wound restitution was also detected after 24 or 48 h of exposure to AMP peptide (Fig. 7), before any mitogenic effect can be detected by an increase in cell number. AMP peptide (Fig. 7B) and rhAMP-18 (not shown) also enhanced migration in nontransformed rat intestinal cells of the IEC-18 line after scrape wounding. This motogenic effect of peptide 77–97 was additive with EGF (Fig. 5, right). Whether there is synergism or not in vivo, the observed additivity suggests that AMP-18 may play an important role in maintaining an intact stomach mucosal epithelium and in facilitating its repair after injury.

DISCUSSION

Previous studies using immunohistochemistry and immunoelectron microscopy suggested that AMP-18 is packaged within secretory granules, which are abundant just under the apical surface of gastric epithelial cells that comprise the antral mucosa (11). The protein

Fig. 7. Time course of the effect of AMP peptide 77–97 on wound restitution in HAE (A) and rat IEC-18 (B) cells. Confluent monolayer cultures were mechanically wounded by scraping with a razor blade, and the distance that cells migrated from the wound edge was measured, as described in Fig. 5 legend. Cells migrated further in the presence of AMP peptide at each time point studied ($P < 0.005$). No difference in cell number was detected in nonwounded cultures in the presence or absence of AMP peptide at 48 h. Values are means ± SE.
appears to be secreted because it was detected (by immunoblotting) in the bathing medium of mouse antral explants and in gastric mucus aspirated from the antrum surface of anesthetized dogs (Fig. 1). Furthermore, decreased AMP-18 content in primary cultures of canine antrum cells exposed to forskolin (Fig. 2) and structurally intact mouse gastric antrum tissue after indomethacin gavage (Fig. 3) suggest that secretion of the protein is subject to regulation. These observations imply that AMP-18 cosecretion with mucus into the viscoelastic gel that overlies the antrum epithelium may be regulated by at least two different signals: an increase in intracellular cyclic AMP and exposure to indomethacin, a nonselective COX inhibitor, but not an agent that is COX-2 selective (rofecoxib). However, the results do not exclude the possibility that exposure to forskolin and/or indomethacin reduces AMP-18 immunoreactivity by stimulating degradation of the protein. Defining the mechanisms by which each of these agents modulates AMP-18 release, degradation, and/or production will require further study.

AMP-18 has now been found to exert pleiotropic effects that enhance mitogenesis and restitution, whether studied as AMP peptide 77–97 (Table 1, Figs. 4 and 5), rhAMP-18 (11), or the native protein in antrum tissue extracts (Fig. 4). As a component of the viscoelastic gel, AMP-18, or possibly a peptide fragment of it in vivo, could protect the antrum epithelium against stresses, such as the action of pepsin, acidic pH, mechanical forces, and high pressures that develop in the gastric lumen during digestion. As a gastric epithelial cell growth factor, AMP-18 could facilitate replenishment of the surface luminal epithelial cell layer to maintain mucosal integrity, if it gains access to the proliferative zone in gastric crypts by back diffusion after injury following damage by nonsteroidal anti-inflammatory drugs, ethanol, or pathogens. After injury of the gastric mucosal surface, restitution occurs very rapidly (8), followed by proliferation and differentiation to reestablish epithelial integrity (15, 20), processes in which AMP-18 and other endogenous molecules (4, 22, 27) could play a role.

Comparison of the predicted secondary structures for AMP-18 proteins of human, pig, and mouse presented in the companion report (11) suggests a conserved helix-loop-sheet domain in a central region now shown to encompass a bioactive peptide, i.e., amino acids 77–101 (Table 1). Studies of peptides within this domain suggest a relatively simple linear model for the growth-stimulatory region: there is an N-terminal extended binding domain (predicted to be largely helical in character, the relative rigidity of which may explain the linear organization of the relevant sequences as determined in the cell growth studies), followed by a region rich in glycine and proline predicted to be a loop structure (Table 1). Although it is unlikely that bioactive peptides assume a stable structure in aqueous solution, we take the conserved predictions to indicate that the structural potentials of this region of AMP-18 may be important for its biological function. It seems reasonable to predict that the interaction of mitogenic peptides with a cell surface receptor could stabilize the active conformation and that the requirement to transiently form the appropriate conformation in solution would explain the lower activity of peptides (21) based on their known amino acid sequence (Table 1). We would explain the specificity of antagonism by peptides 58–68 and 67–85 based on whether they overlapped or not the agonist peptides 58–99 and 84–97; for example 58–68 overlaps and inhibits 58–99, but does not overlap or inhibit 84–97. Finally, only peptide 58–99 (the 42-mer) is recognized by the antisera; peptide 77–97 (a 21-mer) apparently does not contain or cannot form the epitope.

Although a receptor for AMP peptide/AMP-18 has not been identified, data presented in Table 1 are consistent with the hypothesis that peptide-mediated mitogenesis is mediated via a cell surface binding site.
The higher $K_{12}$ value for peptide 84–97 (14-mer) ($K_{12}$: 0.8 μM) than for peptide 58–99 (42-mer) ($K_{12}$: 0.3 μM) (Table 1) suggests that the size and/or sequence of the smaller peptide limits its capacity to bind to a surface site, perhaps due to a reduced ability to form the correct conformation, or possibly because of the loss of ancillary binding regions. The latter notion also is supported by our observations that the nonmitogenic peptides 58–68 and 67–85 can each block the mitogenic activity of peptide 58–99 and the porcine antrum extract (not shown). Finally, peptide 67–85, but not 58–68, antagonizes the activity of peptide 84–97; interestingly, peptide 67–85 overlaps the adjacent 84–97 sequence by two residues.

In summary, AMP-18 may play an important role as a gastrokine in maintaining gastric mucosal integrity and mediating repair after injury, as described for other endogenous proteins synthesized by cells of the GI epithelium, such as trefoil peptides (7, 12, 19, 23, 26) and $\alpha$-defensins (10, 16, 17). Some structural and functional characteristics of these molecules are compared in Table 2. Each of them is secreted by a specific type of GI epithelial cell and differs with regard to the size of its propeptide, mature processed protein, and cDNA. In terms of biological function, only AMP-18 is mitogenic, although it apparently shares with trefoil peptides (i.e., intestinal trefoil factor) the capacity to stimulate restitution, whereas only the $\alpha$-defensin cryptidin 3 is known to induce chloride secretion. Of particular interest is the relatively low concentration (<1 μM) required for either AMP peptide 77–97 or rhAMP-18 protein to exert its biological effects, compared with trefoil peptides and cryptidin 3 (>100 μM), a characteristic more typical of a growth factor or cytokine than a general environmental factor. Additional studies will be required to define these and other roles of AMP-18 in physiological and pathological states.

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