JPET Fast Forward. Published on May 2, 2006 as DOI: 10.1124/jpet.106.102467

JPET #102467

The Cationic Host Defense Peptide rCRAMP Promotes Gastric Ulcer Healing in Rats

Ying H. Yang, William K. K. Wu, Emily K. K. Tai, Helen P. S. Wong, Emily K. Y. Lam, Wallace H. L. So, Vivian Y. Shin, Chi H. Cho

Department of Biology, Hai Nam Normal University, Hai Kou, China (Y. Y.)

Department of Pharmacology (Y. Y., W. W., E. T., H. W., E. L., W. S., V. S., C. C.) and Research Center of Infection and Immunology (C. C.), Faculty of Medicine, The University of Hong Kong, Hong Kong, China

JPET #102467

Running title: Cathelicidin on gastric ulcer healing

Address correspondence to: Chi H. Cho, Department of Pharmacology, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, China. Phone: (852) 2819-9252; Fax: (852) 2817-0859; Email: chcho@hkusua.hku.hk

Number of text pages: 17 (title page, abstract and reference not included)

Number of tables: 0

Number of Figures: 10

Number of References: 40

Number of words in Abstract: 174

Number of words in Introduction: 770

Number of words in Discussion: 831

Non-standard abbreviation:

ADAM, a disintegrin and metalloproteinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal regulated kinase-1/2; HB-EGF, heparin-binding epidermal growth factor-like factor; IL, interleukin; LPS, lipopolysaccharide; MEK, MAPK/ERK kinase; MMP, matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA; TGF, transforming growth factor; VEGF, vascular endothelial growth factor

Recommended section: Gastrointestinal, Hepatic, Pulmonary, and Renal

Abstract

Cathelicidin, a cationic host defense peptide, has been shown to promote cutaneous wound repair and reaches high levels in the gastric mucosa during infection and inflammation. We therefore investigated whether this peptide contributes to gastric ulcer healing in rats. Ulcer induction increased the expression of rat cathelicidin rCRAMP in the gastric mucosa. Further increase in expression of rCRAMP by local injection of rCRAMP-encoding plasmid promoted ulcer healing by enhancing cell proliferation and angiogenesis. rCRAMP directly stimulated proliferation of cultured rat gastric epithelial cells (RGM-1), which was abolished by inhibitors of matrix metalloproteinase (MMP), epidermal growth factor receptors (EGFR) tyrosine kinase, or MAPK/ERK kinase (MEK). rCRAMP also increased EGFR and extracellular signal regulated kinase-1/2 (ERK1/2) phosphorylation via a MMP-dependent mechanism. Knockdown of transforming growth factor α (TGFα), which is a ligand of EGFR, by small interfering RNA completely nullified the mitogenic signals evoked by rCRAMP in RGM-1 cells. These findings suggest that rCRAMP exhibits pro-healing activity in stomachs through TGFα-dependent transactivation of EGFR and its related signaling pathway to induce proliferation of gastric epithelial cells.

Introduction

The gastrointestinal tract is constantly exposed to a repertoire of potentially detrimental agents that may inflict tissue injury. Upon such injury, a repair process is initiated that comprises migration, proliferation, and differentiation of parenchymal and mesenchymal cells in the gastrointestinal mucosa (Podolsky, 1999). Many endogenous molecules that regulate these cellular responses have been identified (Tarnawski et al., 2001; Taupin and Podolsky, 2003). Little studied in this panoply of factors are the host defense peptides, which were originally characterized as small cationic peptides that could kill microbes. This group of peptides is now recognized to contain multifunctional molecules that can modulate many host cellular responses (Li et al., 2000; Selsted and Ouellette, 2005).

Cathelicidins constitute a class of host defense peptides in mammals (Zaiou and Gallo, 2002). They are synthesized as preproprotein, which is characterized by an N-terminal signal sequence, a well-conserved cathelin-like domain, and a C-terminal peptide domain that is proteolytically cleaved to release a small molecular weight host defense peptide. The mature peptides vary markedly among different species. Many mammals such as pigs and cattles have multiple cathelicidin genes, whereas humans, mice, and rats have only one, designated as LL-37/hCAP-18, mCRAMP, and rCRAMP, respectively. The peptide is present in phagocytic granulocytes, the first type of cell to be recruited from the blood to sites of infection and injury, and on surfaces in contact with the outside environment like skin epithelium (Termen et al., 2003). In the gastrointestinal tract, LL-37, the mature peptide of human cathelicidin, is produced constitutively by differentiated surface and upper crypt epithelial cells in the colon and by Brunner's glands in the duodenum (Hase et al., 2002). LL-37 is also

produced in the stomach by surface epithelial cells, as well as chief and parietal cells, and is found in the gastric juice. Recent findings also reveal that LL-37 is upregulated in the gastric secretion and epithelium inflamed by *Helicobacter pylori* (*H. pylori*) infection (Hase et al., 2003).

Wound repair and inflammation are crucial adaptations to tissue damage and bacterial infection. It therefore comes as no surprise that soluble peptide factors have evolved to orchestrate all these processes, including killing bacteria together with regulation of inflammation and wound healing. The human cathelicidin LL-37, in this respect, not only possesses microbicidal activity against a broad spectrum of microorganisms, but also increases chemotaxis of neutrophils, monocytes, T cells, and mast cells (Yang et al., 2000; Niyonsaba et al., 2002). LL-37 also induces secretion of interleukin (IL)-8 and other chemokines from monocytes, macrophages, dendritic cells and epithelial cells and elicits maturation and release of IL-1β in lipopolysaccharide (LPS)-primed monocytes (Scott et al., 2002; Bowdish et al., 2004; Tjabringa et al., 2003; Elssner et al., 2004; Davidson et al., 2004). On the other hand, LL-37 inhibits the expression of specific proinflammatory genes upregulated by LPS, such as NFκB1 (p105/p50) and TNFα-induced protein 2, accompanied by reduced nuclear translocation of NFκb subunits p50 and p65 (Mookherjee et al., 2006). LL-37 also reduces nitric oxide production induced by IL-1β and LPS (Ciornei et al., 2003).

In the context of tissue repair, cathelicidins LL-37 and mCRAMP are strongly expressed in skin epithelium during wound healing in human and mice, respectively (Dorschner et al., 2001). In addition, the expression of LL-37 is low or absent in chronic skin ulcers and antibodies to this peptide inhibit post-wounding re-epithelialization (Heilborn et al., 2003). The ability of LL-37 to induce angiogenesis further highlights its potential role in wound repair (Koczulla et al., 2003). It has been

proposed that the pro-healing effects of cathelicidins may be mediated through modification of growth-factor/receptor interactions and angiogenesis (Li et al., 2000; Chon et al., 2001; Gallo et al., 1994). Definitive proof of the involvement of these mechanisms in wound healing, however, has not yet been obtained. Although *H. pylori*-associated inflammation in the human gastric mucosa is known to induce LL-37 expression, little is known about its role in mucosal repair. In this connection, rCRAMP, the rat cathelicidin, has a similar expression pattern and biological activity to human cathelicidin (Termen et al., 2003; Travis et al., 2000). rCRAMP and LL-37 also share a similar secondary structure, which is characterized by an amphipathic α-helix. It has therefore been proposed that the rat model is an appropriate experimental system to study the role of cathelicidin in human diseases (Termen et al., 2003). Nevertheless, it is also worthwhile to notice that rCRAMP is cytotoxic at high concentration and share stronger homology to mCRAMP than to LL-37 (Termen et al., 2003). In the present study, we aim to investigate whether this peptide promotes gastric ulcer healing in rats through a defined mitogenic mechanism in gastric epithelial cells.

Methods

Reagents and drugs

The synthetic mature rCRAMP peptide and antiserum against rCRAMP were purchased from Innovagen (Lund, Sweden). The peptide had the following amino acid sequence: NH_2-RFKKISRLAG LLRKGGEKFG EKLRKIGQKI KDFFQKLAPE IEQ-COOH. The broad specificity matrix metalloproteinase (MMP) inhibitor GM6001 (N-[(2R)-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan Methylamide), epidermal growth factor receptor (EGFR) kinase inhibitor AG1478 (4-(3-Chloroanilino)-6,7-dimethoxyquinazoline), and MAPK/ERK kinase (MEK)-specific inhibitor U0126 (1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene) were from Calbiochem (San Diego, CA). Antibodies to total and phosphorylated extracellular signal regulated kinase-1/2 (ERK1/2) were purchased from Cell Signaling Technology (Beverley, MA). Antibodies to EGFR and anti-phosphotyrosine antibody (4G10) were from Upstate Biotechnology (Lake Placid, NY). All other chemicals and reagents were from Sigma (St Louis, MO) unless otherwise specified.

Induction of experimental ulcer and sample collection

The study was approved by the Committee on the Use of Live Animals for Teaching and Research at the University of Hong Kong. Male Sprague-Dawley rats (180-200 g) were reared on a standard laboratory diet (Ralston Purina, Chicago, IL) and given tap water. Rats were deprived of food but had free access to tap water 24 h before ulcer induction. Gastric kissing ulcers were produced by luminal application of acetic acid. Animals were sacrificed on days 1, 4, 7 and 10 after ulcer induction and

the ulcer area was measured as previously described (Ma et al., 2000), by a person who was unaware of the type of treatment. After measuring the ulcerated area, a longitudinal section of stomach along the greater curvature, including the ulcer base and both sides of the ulcer margin, was fixed in 4% buffered formalin for 24 h at 4°C for histologic study. The remaining glandular mucosa around the ulcer (including the ulcer margin and adjacent normal mucosa) was scraped with a glass slide on an ice-cold dish and immediately frozen in liquid nitrogen. The mucosal samples were stored at -70°C for isolation of RNA and protein.

Gene therapy with rCRAMP plasmid

Full-length rCRAMP complementary DNA was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) under a CMV promoter. The same plasmid without the rCRAMP insert was used as control. All plasmids were amplified in DH5α *E. coli*-competent cells (Invitrogen, Carlsbad, CA) and purified with an endo-free plasmid mega-prep kit (Qiagen, Valencia, CA). Twenty-four male Sprague-Dawley rats were fasted for 24 h before experiments. Gastric ulcers were induced as described earlier. Immediately after ulcer induction, each of 12 rats were injected with 100 μg of the plasmid DNA with rCRAMP insert and the remaining rats were injected with the same amount of control plasmid DNA. The plasmid DNA was injected from 4 sides of the ulcer induction site into the submucosa as described by Jones *et al* (Jones et al., 2001).

Histology and immunohistochemistry

Sections were stained for proliferative cells and microvessels by immunohistochemistry as previously described (Shin et al., 2004). To assess cell

proliferation, sections were digested with trypsin for 15 min at room temperature and incubated with a blocking agent (LSAB kit, DAKO, Copenhagen, Denmark) for 1 h. They were then incubated with a monoclonal primary antibody against mouse proliferating cell nuclear antigen (PCNA) (1:200) overnight at 4°C. Sections were incubated with Link reagent (LSAB kit) for 1 h, followed by streptavidin for another hour. Finally they were incubated with hydrogen-peroxidase-diaminobenzidine to visualize PCNA-positive cells. After washing with tap water, sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. The number of stained cells was counted under a microscope at 400× magnification. Microvessel density was measured with a procedure similar to PCNA-staining, except that rabbit anti-human von Willebrand factor (1:200) (DAKO) was used to identify microvessels, and Mayer's hematoxylin counterstaining was omitted. Microvessel density was expressed as the number of microvessels per mm^2 in 5-8 randomly selected fields (×200).

Cell culture and viability assay

Rat gastric mucosal epithelial cell line RGM-1 (RCB-0876, Riken Cell Bank, Tsukuba, Japan) was grown in DMEM/F-12 medium (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 20% FBS (GIBCO, Gaitherburg, MD) in an incubator at 37°C, 95% humidity, and 5% CO_2. Cell viability was measured using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method as previously described (Shin et al., 2004).

[^{3}H]Thymidine incorporation assay

JPET Fast Forward. Published on May 2, 2006 as DOI: 10.1124/jpet.106.102467
This article has not been copyedited and formatted. The final version may differ from this version.

Cell proliferation was assessed as DNA synthesis by the [^{3}H]thymidine incorporation assay with modifications. RGM-1 cells were seeded in 24-well culture plates at ~5×10^4 cells/ml and were allowed to grow in DMEM/F-12 medium containing 20% FBS for 24 h. Afterwards, cells were growth arrested in serum-free medium overnight, then various concentrations of rCRAMP (5-20 μg/ml) were incubated with the cells for 24 h to study the mitogenic effect of rCRAMP in the presence or absence of AG1478 (1 μM), U0126 (25 μM), or GM6001 (25 μM), which were administered 30 min prior to rCRAMP treatment. In the next step, 0.5 μCi of [^{3}H]thymidine was added to each well, and the cells were incubated for a further 4 h. The final incorporation of [^{3}H]thymidine into cells was measured with a liquid scintillation counter (LS-6500, Beckman Instruments, Inc., Pullerton, CA).

Immunoprecipitation and Western blot analysis

RGM-1 cells were harvested in radioimmunoprecipitation assay (RIPA) buffer containing proteinase and phosphatase inhibitors as described previously (Shin et al., 2004). Protein was quantified with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). To immunoprecipitate EGFR, lysates (500 μg) were incubated overnight at 4°C with 1 μg of anti-EGFR antibody and 20 μl of a 50% protein A-Sepharose slurry. Beads were washed with RIPA buffer three times. They were then boiled in 2× Laemmli sample buffer and run on SDS polyacrylamide gels. For the Western blot analysis, equal amounts of protein (40 μg/lane) were resolved by SDS-PAGE, and transferred to Hybond C nitrocellulose membranes (Amersham, Arlington Heights, IL). For mature rCRAMP, SDS-PAGE was performed using 16.5% tricine gels. Membranes were probed with anti-EGF, anti- VEGF, anti-ERK1/2, anti-phospho-ERK1/2, anti-EGFR, and anti-phosphotyrosine antibodies overnight at 4°C

and incubated for 1 h with secondary peroxidase-conjugated antibodies. The proteins were visualized with an enhanced chemiluminescence system (Amersham).

RT-PCR for rCRAMP, TGFα, and β-actin

The total RNA was isolated from rat gastric tissues and RGM-1 cells with TriZol™ reagent (Invitrogen). Two micrograms of total RNA were reverse-transcribed using the Thermoscript™ RT-PCR system (Invitrogen). PCR was performed for rCRAMP, TGFα, and β-actin using the following primer pairs: rCRAMP, sense primer 5'- TCTGAGCCCCAAGGGGATGAGGA-3' and antisense primer 5'-CCAAGGCAGGCCTACTGCTCTAT-3' (product size 356 bp); TGFα, sense primer 5'-CTGGGTATCCTGGTAGCTGTGT-3' and antisense primer 5'-GACCACTGTCTCAGAGTGGC-3' (product size 322 bp); and β-actin, sense primer 5'-GTGGGGCGCCCCAGGCACCA-3' and antisense primer 5'-CTCCTTAATGTCACGCACGATTTC-3' (product size 540 bp). The PCR conditions were as follows: the template cDNA was first denatured at 94°C for 5 min. During 40 cycles of amplification, the denaturation step was at 94°C for 1 min, the annealing step at 55°C for 1 min and the extension step at 72°C for 1 min. The final extension step was at 72°C for 7 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. Gel photographs were then analyzed semiquantitatively in a multianalyzer (Bio-Rad Laboratories, Hercules, CA).

Knockdown of TGFα by small interfering RNA

siRNA targeting rat TGFα (TGFα-siRNA) was designed online and synthesized by Invitrogen. The sequence of TGFα-siRNA, which corresponded to the coding regions 139 to 164 relative to the first nucleotide of the start codon, were as

JPET Fast Forward. Published on May 2, 2006 as DOI: 10.1124/jpet.106.102467
This article has not been copyedited and formatted. The final version may differ from this version.

follows: 5'-CAGAUUCCCACACUCAGUAUUGUUU-3' and 5'-AAACAAUACUGAGUGUGGGAAUCUG-3'. Transfection was performed using Oligofectamine™ Reagent (Invitrogen) according to the manufacturer's instructions. The efficacy of TGFα knockdown was assessed by RT-PCR. Assays were performed 2 days after transfection.

Statistical analysis

Results were expressed as the mean ± SEM. Statistical analysis was performed with ANOVA followed by the Tukey t-test. P values <0.05 were considered statistically significant.

Results

Gastric ulceration induced rCRAMP expression

To determine if ulceration altered rCRAMP expression in rat gastric epithelium, mucosal biopsies at various time points after ulcer induction were evaluated for expression of the peptide. rCRAMP mRNA was detected at low levels in normal gastric tissues, but was significantly upregulated by 84% and 93% in ulcerated gastric tissue on days 1 and 4 after ulcer induction, respectively. In parallel with ulcer healing, rCRAMP mRNA declined in the mucosa on day 7 and had almost returned to basal levels on day 10 (Figure 1A). Protein levels of the mature form of rCRAMP demonstrated a similar pattern of changes in the gastric mucosa both in the basal condition and during ulcer healing (Figure 1B).

Gene therapy with rCRAMP plasmid accelerated gastric ulcer healing

Local injection of rCRAMP plasmid was performed to overexpress this peptide around the ulcer site. On day 4, in rats with ulcerated stomach, gene therapy increased both mRNA (Figure 2A) and protein (Figure 2B) levels of rCRAMP by 97% and 83% respectively ($P < 0.05$, $N = 6$) compared with those injected with control plasmid. Gene therapy also significantly reduced ulcer size by 49.8% ($P < 0.01$) on day 4. The average ulcerated area was 38.3 mm^2 in the gene therapy group versus 80.4 mm^2 in the plasmid control group (Figure 2C).

Overexpression of rCRAMP promoted epithelial cell proliferation and angiogenesis *in vivo*

The immunoreaction for PCNA was observed as a dark accumulation of diaminobenzidine reaction products in the nuclei of the epithelial cells in the ulcer margin and in fibroblasts in the granulation tissue. The number of PCNA-positive cells at the ulcer margin was significantly higher in the rCRAMP plasmid-treated animals ($P < 0.05$) than in control animals (52 ± 2.5 versus 34 ± 3.0) (Figure 3A). The number of microvessels in rats treated with rCRAMP plasmid was also higher than that of the control group (29 ± 2.0 versus 17 ± 1.2) (Figure 3B). To investigate whether rCRAMP influenced proliferation and angiogenesis by induction of EGF and/or VEGF synthesis (Ma et al., 2000; Jones et al., 1999), we determined the protein levels of these factors in the gastric mucosa on day 4 after ulcer induction and found that their expression was not affected by the rCRAMP gene therapy (data not shown).

Synthetic rCRAMP stimulated proliferation of cultured gastric epithelial cells

Given the marked increase in the number of proliferative cells at the ulcer margin induced by rCRAMP plasmid treatment, we examined further whether rCRAMP directly affected the growth of cultured rat gastric epithelial cells (RGM-1). We found that rCRAMP treatment significantly induced cell proliferation in a dose-dependent manner. At 5 and 10 μg/ml, the peptide increased cell proliferation by 20.5% and 36.1% respectively. EGF was used as a positive control, which stimulated cell proliferation by 29.4% (Figure 4). At all concentrations used, rCRAMP exerted no cytotoxic effect on the gastric epithelial cells.

Synthetic rCRAMP increased ERK1/2 and EGFR phosphorylation

Because the ERK1/2 signaling pathway can be involved in cell proliferation (Pai et al., 1998), the effect of rCRAMP on ERK1/2 activation in RGM-1 cells was studied. Results demonstrated that rCRAMP induced a concentration-dependent increase in ERK1/2 phosphorylation (Figure 5A) that was significant over the concentration range of 5-20 μg/ml, and maximal at 10 μg/ml. In cells treated with 10 μg/ml rCRAMP, ERK1/2 phosphorylation was maximal at 15 min after incubation, and returned to the basal level at 60 min (Figure 5B). In addition, activation of ERK1/2 by rCRAMP was fully inhibited by pretreatment with MAPK/ERK (MEK) inhibitor U0126 (Fig. 5C). Treatment of RGM-1 cells with rCRAMP also caused an increase in tyrosine phosphorylation of epidermal growth factor receptor (EGFR) which was completely abolished by AG1478 (an EGFR kinase inhibitor) (Figure 6A). Treatment of RGM-1 cells with AG1478, moreover, dramatically decreased the phosphorylation of ERK1/2 induced by rCRAMP without altering total protein levels of ERK1/2 (Figure 6B).

MMP inhibitor abolished rCRAMP-induced EGFR and ERK1/2 phosphorylation

As rCRAMP was unlikely to bind and activate EGFR directly, we hypothesized that this event could be mediated by EGFR transactivation which requires the cleavage of membrane-bound EGFR ligands by MMP. To this end, treatment of RGM-1 cells with MMP inhibitor GM6001 significantly inhibited EGFR and ERK1/2 phosphorylation induced by rCRAMP (Figure 7). As a positive control, GM6001 had no effect on the EGFR phosphorylation mediated by EGF.

Inhibitors of MMP, EGFR tyrosine kinase, and MEK abolished the mitogenic effect of rCRAMP

To determine the involvement of MMP, EGFR, and MEK in rCRAMP-induced cell proliferation, cell proliferation was assayed in the absence or presence of respective inhibitors. Results revealed that inhibition of MMP, EGFR tyrosine kinase, and MEK with GM6001, AG1478, and U0126 respectively abolished rCRAMP-induced cell proliferation (Figure 8), indicating that rCRAMP-induced cell proliferation was dependent on these factors.

TGFα knockdown nullified the effect of rCRAMP on EGFR phosphorylation and cell proliferation

Although the results presented so far clearly indicated that rCRAMP induced cell proliferation through EGFR transactivation, whether this phenomenon is ligand-dependent or not had not yet been determined. It has been reported that gastric mucosa and gastric cancer cell lines produce heparin-binding-EGF-like growth factor (HB-EGF), TGFα, and amphiregulin, all of which can be mobilized by MMP and thereby activate EGFR (Tanida et al., 2004; Naef et al., 1996). In RGM-1 cells, the mRNAs of TGFα and amphiregulin, but not HB-EGF, were detected by RT-PCR. The expression level of TGFα was higher than that of amphiregulin (data not shown). Furthermore, downregulation of TGFα by small interfering RNA (siRNA) significantly suppressed rCRAMP-induced EGFR phosphorylation (Figure 9, A and B) and cell proliferation (Figure 9C). These findings are in accordance with the speculation that rCRAMP-induced EGFR transactivation is dependent on the release of TGFα. Furthermore, knockdown of TGFα decreased basal DNA synthesis by

JPET Fast Forward. Published on May 2, 2006 as DOI: 10.1124/jpet.106.102467
This article has not been copyedited and formatted. The final version may differ from this version.

approximately 15% ($P < 0.05$), indicating that TGFα is required for the maintenance of basal levels of proliferation in RGM-1 cells.

Discussion

In mammals, several host defense peptides, such as β-defensins and cathelicidins, serve important innate immune functions by acting as 'natural antibiotics' to provide first-line defense against infection (Selsted et al., 2005; Elsbach, 2003; Schutte and McCray, 2002). These peptides are expressed on epithelial surfaces and in neutrophils, and are upregulated upon bacterial infection. In the present study, we demonstrated for the first time that the rat cathelicidin rCRAMP was involved in tissue repair in the stomach. To this end, ulceration upregulated the expression of rCRAMP in the gastric mucosa. Further induction of rCRAMP expression by plasmid-based gene therapy accelerated ulcer healing by promoting angiogenesis and cell proliferation in the gastric mucosa. In accord with these findings, human cathelicidin LL-37 has been reported to promote proliferation and migration of human airway epithelial cells as well as cutaneous wound repair. In addition, LL-37 is known to directly induce proliferation and formation of vessel-like structures in cultivated endothelial cells. Mice deficient in mCRAMP also exhibit significantly decreased vascular structures and delayed wound closure (Heilborn et al., 2003; Koczulla et al., 2003; Shaykhiev et al., 2005). All these findings suggest that cathelicidin is an important endogenous mitogenic and pro-angiogenic factor in gastric ulcer healing. However, the mechanism by which ulceration upregulates rCRAMP expression in the gastric mucosa is presently unknown. Moreover, whether rCRMAP, a known modulator of inflammation (Yang et al., 2000; Niyonsaba et al., 2002; Mookherjee et al., 2006), can possibly alter the cytokine environment and thereby facilitate the subsequent healing process remains unexplored. In this regard, our results show that the upregulation of rCRAMP could be detected early after ulcer induction (on day 1

and day 4), implicating that rCRAMP might be involved in the modulation of acute inflammatory response during gastric ulceration.

Previous studies demonstrated that EGFR and its related signalling pathway are involved in the proliferation of gastric epithelial cells (Pai et al., 1998; Tarnawski et al., 1992). Here we report that rCRAMP increased RGM-1 cell proliferation as well as EGFR and ERK1/2 phosphorylation, which was abolished by inhibition of MMP or knockdown of TGFα, indicating that the mitogenic action of rCRAMP is signalling through MMP-mediated TGFα-dependent transactivation of EGFR. In addition, the MMP inhibitor had no effects on the EGF-induced phosphorylation of EGFR and ERK1/2, suggesting that activation of the EGFR and ERK1/2 pathway by rCRAMP may involve cleavage of membrane-anchored EGFR ligands by MMP. In this regard, it is known that EGFR ligands such as TGFα and HB-EGF are synthesized as transmembrane precursor molecules, which can be cleaved by cell surface proteinases known as ADAM (a disintegrin and metalloproteinase) to release the soluble form, a process known as ectodomain shedding (Xiao and Majumdar, 2001; Sahin et al., 2004). It is therefore believed that the mitogenic signal evoked by rCRAMP in gastric epithelial cells is effected by ADAM-mediated ectodomain shedding of TGFα, which can be blocked by MMP inhibitors (Figure 10). In this context, ADAM17 has been shown to be the major convertase responsible for the shedding of TGFα (Sahin et al., 2004). Indeed, it has been demonstrated that LL-37 can transactivate EGFR via metalloproteinase-mediated cleavage of membrane-anchored EGFR-ligands to induce IL-8 release (Tjabringa et al., 2003). Prostaglandin E_2 also signals through a similar pathway to transactivate EGFR and phosphorylate ERK1/2 by stimulating MMP-dependent cleavage of TGFα in colon cancer and gastrointestinal hypertrophy (Pai et al., 2002). The molecular mechanisms underlying cathelicidin-induced activation of

MMP, however, are presently unknown but may involve signals mediated by a G-protein-coupled receptor (Shaykhiev et al., 2005).

Recently, antimicrobial peptides have generated intense interest because of their therapeutic potential against antibiotic-resistant pathogens. There is also a rising concern over *H. pylori* resistance to antibiotics. For instance, *H. pylori* resistance to macrolide, metronidazole, amoxicillin, and tetracycline are increasingly reported and may limit the efficacy of current treatment (Megraud, 2004). Second-line therapy for *H. pylori* eradication is therefore promptly needed. To this end, cathelicidin has been shown to be bactericidal for several strains of *H. pylori*, including SD4, SD14, and SS1 (Hase et al., 2003). In the current study, we also demonstrate that overexpression of cathelicidin can promote ulcer healing in rats. Cathelicidin may therefore be a potential natural antibiotic for the eradication of *H. pylori* in gastric ulcer patients while at the same time promoting ulcer repair in the gastric mucosa. Although direct administration of synthesized peptide seems unlikely due to its poor chemical stability in the stomach, endoscopic injection of cathelicidin-encoding plasmids or inoculation of bioengineered bacteria that actively produce cathelicidin (e.g. *Lactobacillus* transformed with cathelicidin-encoding plasmids) could be a clinically effective method for delivery of cathelicidin at the ulcer site (Steidler et al., 2000; Jones et al., 2001).

To conclude, our experimental findings not only establish a novel role for cathelicidin in ulcer healing, but also define the mechanistic pathways of its mitogenic action on gastric epithelial cells. This unique and naturally produced protein that combines antimicrobial and ulcer healing actions may provide a new therapeutic approach in the treatment of infectious gastric ulcer.

References

Bowdish DM, Davidson DJ, Speert DP and Hancock RE (2004) The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J Immunol* **172**:3758-3765.

Chon JH, Houston MM, Xu C and Chaikof EL (2001) PR-39 coordinates changes in vascular smooth muscle cell adhesive strength and locomotion by modulating cell surface heparan sulfate-matrix interactions. *J Cell Physiol* **189**:133-143.

Ciornei CD, Egesten A and Bodelsson M (2003) Effects of human cathelicidin antimicrobial peptide LL-37 on lipopolysaccharide-induced nitric oxide release from rat aorta in vitro. *Acta Anaesthesiol Scand* **47**:213-220.

Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, Hancock RE and Speert DP (2004) The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J Immunol* **172**:1146-1156.

Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, Agerberth B, Gudmundsson GH and Gallo RL (2001) Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J Invest Dermatol* **117**:91-97.

Elsbach P (2003) What is the real role of antimicrobial polypeptides that can mediate several other inflammatory responses? *J Clin Invest* **111**:1643-1645.

Elssner A, Duncan M, Gavrilin M and Wewers MD (2004) A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol* **172**:4987-4994.

Gallo RL, Ono M, Povsic T, Page C, Eriksson E, Klagsbrun M and Bernfield M (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc Natl Acad Sci U S A* **91**:11035-11039.

Hase K, Eckmann L, Leopard JD, Varki N and Kagnoff MF (2002) Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. *Infect Immun* **70**:953-963.

Hase K, Murakami M, Iimura M, Cole SP, Horibe Y, Ohtake T, Obonyo M, Gallo RL, Eckmann L and Kagnoff MF (2003) Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. *Gastroenterology* **125**:1613-1625.

Heilborn JD, Nilsson MF, Kratz G, Weber G, Sorensen O, Borregaard N and Stahle-Backdahl M (2003) The cathelicidin anti-microbial peptide LL-37 is

involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J Invest Dermatol* **120**:379-389.

Jones MK, Itani RM, Wang H, Tomikawa M, Sarfeh IJ, Szabo S and Tarnawski AS (1999) Activation of VEGF and Ras genes in gastric mucosa during angiogenic response to ethanol injury. *Am J Physiol* **276**:G1345-G1355.

Jones MK, Kawanaka H, Baatar D, Szabo IL, Tsugawa K, Pai R, Koh GY, Kim I, Sarfeh IJ and Tarnawski AS (2001) Gene therapy for gastric ulcers with single local injection of naked DNA encoding VEGF and angiopoietin-1. *Gastroenterology* **121**:1040-1047.

Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, Issbrucker K, Unterberger P, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Vogelmeier C, Gallo RL, Clauss M and Bals R (2003) An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* **111**:1665-1672.

Li J, Post M, Volk R, Gao Y, Li M, Metais C, Sato K, Tsai J, Aird W, Rosenberg RD, Hampton TG, Sellke F, Carmeliet P and Simons M (2000) PR39, a peptide regulator of angiogenesis. *Nat Med* **6**:49-55.

Ma L, Wang WP, Chow JY, Yuen ST and Cho CH (2000) Reduction of EGF is associated with the delay of ulcer healing by cigarette smoking. *Am J Physiol Gastrointest Liver Physiol* **278**:G10-G17.

Megraud F (2004) H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* **53**:1374-1384.

Mookherjee N, Brown KL, Bowdish DM, Doria S, Falsafi R, Hokamp K, Roche FM, Mu R, Doho GH, Pistolic J, Powers JP, Bryan J, Brinkman FS, and Hancock RE (2006) Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J Immunol* 176:2455-2464.

Naef M, Yokoyama M, Friess H, Buchler MW and Korc M (1996) Co-expression of heparin-binding EGF-like growth factor and related peptides in human gastric carcinoma. *Int J Cancer* **66**:315-321.

Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H and Nagaoka I (2002) A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* **106**:20-26.

Pai R, Ohta M, Itani RM, Sarfeh IJ and Tarnawski AS (1998) Induction of mitogen-activated protein kinase signal transduction pathway during gastric ulcer healing in rats. *Gastroenterology* **114**:706-713.

Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D and Tarnawski AS (2002) Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* **8**:289-293.

Podolsky DK (1999) Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *Am J Physiol* **277**:G495-G499.

Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P and Blobel CP (2004) Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol* **164**:769-779.

Schutte BC and McCray PB Jr (2002) [beta]-defensins in lung host defense. *Annu Rev Physiol* **64**:709-748.

Scott MG, Davidson DJ, Gold MR, Bowdish D and Hancock RE (2002) The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol* **169**:3883-3891.

Selsted ME and Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. *Nat Immunol* **6**:551-557.

Shaykhiev R, Beisswenger C, Kaendler K, Senske J, Puechner A, Damm T, Behr J and Bals R (2005) The human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am J Physiol Lung Cell Mol Physiol* **289**:L842-L848.

Shin VY, Wu WK, Ye YN, So WH, Koo MW, Liu ES, Luo JC and Cho CH (2004) Nicotine promotes gastric tumor growth and neovascularization by activating

extracellular signal-regulated kinase and cyclooxygenase-2. *Carcinogenesis* **25**:2487-2495.

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W and Remaut E (2000) Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science* **289**:1352-1355.

Tanida S, Joh T, Itoh K, Kataoka H, Sasaki M, Ohara H, Nakazawa T, Nomura T, Kinugasa Y, Ohmoto H, Ishiguro H, Yoshino K, Higashiyama S and Itoh M (2004) The mechanism of cleavage of EGFR ligands induced by inflammatory cytokines in gastric cancer cells. *Gastroenterology* **127**:559-569.

Tarnawski A, Stachura J, Durbin T, Sarfeh IJ and Gergely H (1992) Increased expression of epidermal growth factor receptor during gastric ulcer healing in rats. *Gastroenterology* **102**:695–698.

Tarnawski A, Szabo IL, Husain SS and Soreghan B (2001) Regeneration of gastric mucosa during ulcer healing is triggered by growth factors and signal transduction pathways. *J Physiol Paris* **95**:337-344.

Taupin D and Podolsky DK (2003) Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* **4**:721-732.

Termen S, Tollin M, Olsson B, Svenberg T, Agerberth B and Gudmundsson GH (2003) Phylogeny, processing and expression of the rat cathelicidin rCRAMP: a model for innate antimicrobial peptides. *Cell Mol Life Sci* **60**:536-549.

Tjabringa GS, Aarbiou J, Ninaber DK, Drijfhout JW, Sorensen OE, Borregaard N, Rabe KF and Hiemstra PS (2003) The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J Immunol* **171**:6690-6696.

Travis SM, Anderson NN, Forsyth WR, Espiritu C, Conway BD, Greenberg EP, McCray PB Jr, Lehrer RI, Welsh MJ and Tack BF (2000) Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect Immun* **68**:2748-2755.

Xiao ZQ and Majumdar AP (2001) Increased in vitro activation of EGFR by membrane-bound TGF-alpha from gastric and colonic mucosa of aged rats. *Am J Physiol Gastrointest Liver Physiol* **281**:G111-G116.

Yang D, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ and Chertov O (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, uses formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes and T cells. *J Exp Med* **192**:1069-1074.

Zaiou M and Gallo RL (2002) Cathelicidins, essential gene-encoded mammalian antibiotics. *J Mol Med* **80**:549-561.

Footnotes

Financial support: The Committee on Research and Conference Grants of the University of Hong Kong and The Hong Kong Research Grants Council (HKU 7397/03M).

Request for reprint: Chi H. Cho, Department of Pharmacology, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, China. Phone: (852) 2819-9252; Fax: (852) 2817-0859; Email: chcho@hkusua.hku.hk

[1] Ying H. Yang and William K. K. Wu contributed equally to the paper

Figure legends

Fig. 1. rCRAMP expression during gastric ulcer healing. (A) RT-PCR showed increased rCRAMP mRNA expression at days 1, 4, and 7 after ulcer induction. RT-PCR products were obtained with specific primers that recognized full length rCRAMP mRNA (356 bp) and primers that recognized rat ß-actin; (B) Western blot showed increased mature rCRAMP peptide expression at days 1, 4, 7, and 10.

Fig. 2. rCRAMP overexpression accelerated ulcer healing on day 4 and day 7. (A) Western blot with anti-rCRAMP serum showed an increase in mature rCRAMP peptide in the gastric mucosa in rCRAMP-plasmid injected rats. (B) Rats injected locally with rCRAMP expression plasmid showed significant reduction of ulcer size compared with control plasmid-injected rats on day 4 and day 7. *, P <0.05 versus corresponding control group.

Fig. 3. Effects of overexpression of rCRAMP on (A) mucosa cell proliferation on day 4 and day 7 and (B) angiogenesis in gastric ulcers on day 4 after ulcer induction and injection of either control plasmid or rCRAMP plasmid (n = 6). Mucosal cell proliferation was assessed by counting the number of PCNA-positive cells. The numbers of microvessels counted in five microscopic fields in the ulcer base at ×200 were averaged. *, P <0.05 compared with the corresponding control group.

Fig. 4. Synthetic rCRAMP induced proliferation in RGM cells**.** Subconfluent RGM-1 cells were serum-starved overnight and stimulated with 0-20 µg/ml rCRAMP or 20 pg/ml EGF for 24 h. For the final 4 h of stimulation, [^{3}H]thymidine was added to the medium for labeling. Data are presented as mean ± SEM of two triplicate experiments. *, P <0.05; **, P <0.01 versus control group.

Fig. 5. Activation of ERK1/2 by rCRAMP. (A) Concentration-dependent activation of ERK1/2 by rCRAMP. RGM-1 cells were treated with 0-20 μg/ml rCRAMP for 15 min. Total cell lysates were assessed for activity by Western blotting with an antibody against the dually phosphorylated form of ERK1/2. An immunoblot of total ERK served as a loading control. Quantitative analysis of phospho-ERK1/2 normalized against the corresponding total ERK1/2 signal. (B) Time course of ERK activation by rCRAMP. RGM cells were treated with 10 μg/ml rCRAMP for the indicated times. Cells were lysed, and the soluble fraction was blotted for phosphorylated ERK or total ERK as loading control. The quantitative analysis of phospho-ERK was normalized to zero time. (C) RGM-1 cells were pretreated with U0126 (20 μM) for 30 min where indicated before stimulating with or without 10 μg/ml rCRAMP for 15 min. Cell extracts were analyzed for activation of ERK1/2. The quantitative analysis of phospho-ERK was normalized to total ERK1/2 signal. Results represent means ± SEM from three independent experiments.*, P <0.05 versus control; $^{\#}$, P <0.05 versus rCRAMP-treated group.

Fig. 6. rCRAMP transactivated EGFR and triggered the mitogenic ERK1/2 signaling pathway in RGM-1. (A) Transactivation of EGFR by rCRAMP. RGM-1 cells were treated with vehicle or AG1478 (1 μM) followed by incubation with or without rCRAMP (10 μg/ml) for 15 min. After lysis, EGFR was immunoprecipitated with anti-EGFR antibody and tyrosine-phosphorylated EGFR was detected by immunoblotting with anti-phosphotyrosine antibody. The total amount of EGFR in immunoprecipitates was determined by Western blot analysis with antibodies to the

EGFR (upper panel). Quantitative analysis of EGFR phosphorylation normalized to control from 3 separate experiments (means ± SEM) (lower panel). (B) RGM-1 cells were serum-starved overnight, and then treated with AG1478 (1 μM) for 30 min prior to rCRAMP (10 μg/ml) stimulation for 15 min, and total cellular protein was collected. An equal amount of protein was separated by SDS-PAGE, and phosphorylated ERK was visualized with anti-phospho-ERK antibodies. Equal loading of lysate is shown by Western analysis with total ERK antibodies (upper panel). Quantitative analysis of ERK1/2 phosphorylation normalized to control from 3 separate experiments (means ± SEM) (lower panel). *, P <0.05 versus control; $^{\#}$, P <0.05 versus rCRAMP-treated group.

Fig. 7. Matrix metalloproteinase(s) (MMP) activity is required for rCRAMP-mediated EGFR transactivation and ERK1/2 signaling. RGM-1 cells were pretreated with either vehicle, GM6001 (25 μM) for 30 min prior to stimulation with rCRAMP (10 μg/ml) for 15 min or EGF for 2 min. (A) EGFR was immunoprecipitated with anti-EGFR antibody and tyrosine-phosphorylated EGFR was detected by immunoblotting with anti-phosphotyrosine antibody (upper panel). The total amount of EGFR was determined by Western blot with anti-EGFR antibody with equal loading of immunoprecipitates (lower panel). (B) Activation of ERK1/2 was followed by Western blotting with anti-phospho-ERK1/2 antibodies (upper panel). Equal loading of lysate is shown by Western analysis with anti-total ERK1/2 antibodies (lower panel).

Fig. 8. Effect of inhibitors of MMP, EGFR, and MEK on rCRAMP-induced proliferation in RGM-1 cells. Cells were pre-incubated for 1 h with the following

inhibitors before adding 10 μg/ml rCRAMP: medium alone, the EGFR tyrosine kinase inhibitor AG1478 (1 μM), the MEK inhibitor U0126 (25 μM), and the metalloproteinase inhibitor GM6001 (25 μM). As a control, cells were incubated with medium alone. After 24 h, cell proliferation was determined by [^{3}H]thymidine incorporation. Results are expressed as ± SEM of one representative experiment of three, each performed in triplicate. *, P <0.05 versus control group; $^{\#}$, P <0.05 versus rCRAMP-treated group.

Fig. 9. Effects of knockdown of TGFα by siRNA on rCRAMP-induced EGFR phosphorylation and cell proliferation. (A) Effects of knockdown of endogenous TGFα mRNA by specific siRNA. RGM-1 cells were treated with siRNA targeting TGFα and the levels of TGFα transcript were detected by RT-PCR. (B) Effects of knockdown of TGFα on rCRAMP-induced EGFR phosphorylation. RGM-1 cells incubated with rCRAMP (10 μg/ml) for 15 min after exposure to control or TGFα siRNA were lysed and immunoprecipitated (IP) for EGFR. The immunoprecipitate was analyzed by Western Blot (WB) using anti-phosphortyrosine (PY) or anti-EGFR antibody. (C) Effects of knockdown of TGFα on rCRAMP-induced cell proliferation. RGM-1 cells were treated with or without siRNA targeting TGFα for 48 h, then incubated with or without rCRAMP for another 24 h. Cell proliferation was determined by [^{3}H]thymidine incorporation. Results are expressed as mean ± SEM from three experiments, each performed in triplicate. **, P <0.01 versus corresponding vehicle group; $^{\#}$, P <0.05 versus vehicle group treated with control siRNA.

Fig 10. Proposed mechanism for the mitogenic action of rCRMAP in gastric epithelial cells. Receptor activation results in the activation of ADAM, ectodomain shedding of

TGFα, EGFR transactivation, and subsequent ERK1/2 phosphorylation, which results in cell proliferation.

Figure 1

Figure 2

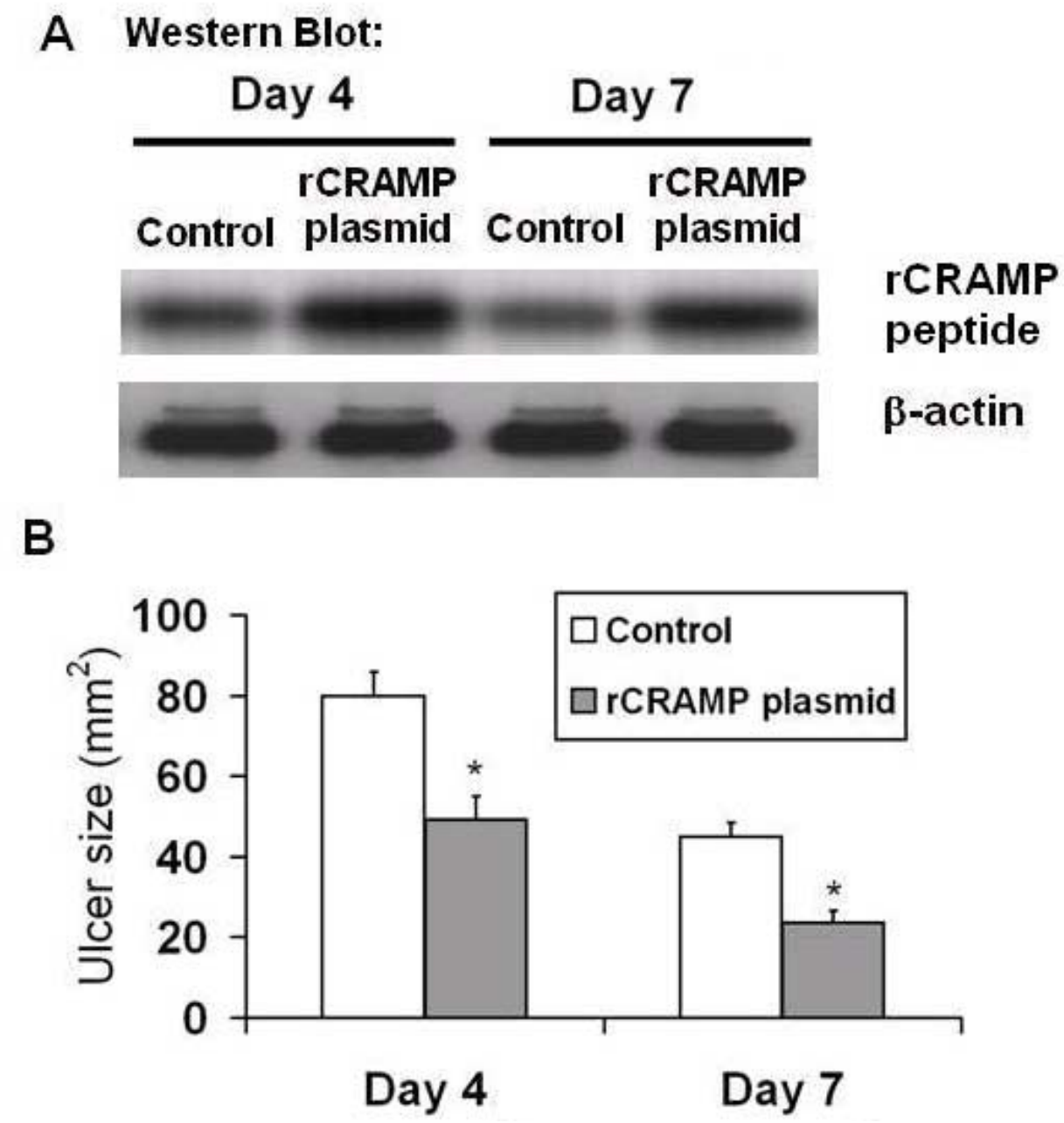


Figure 3

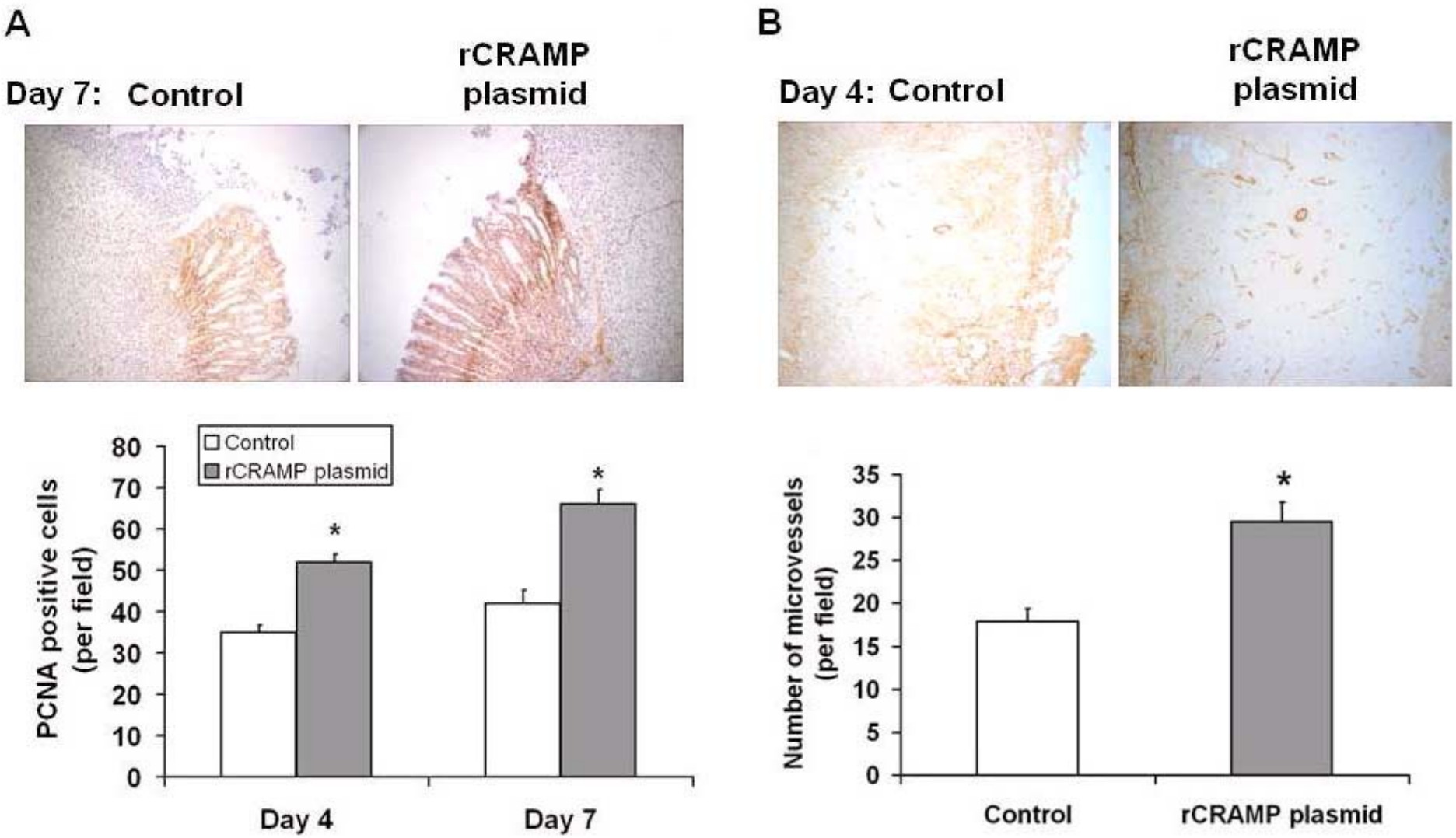
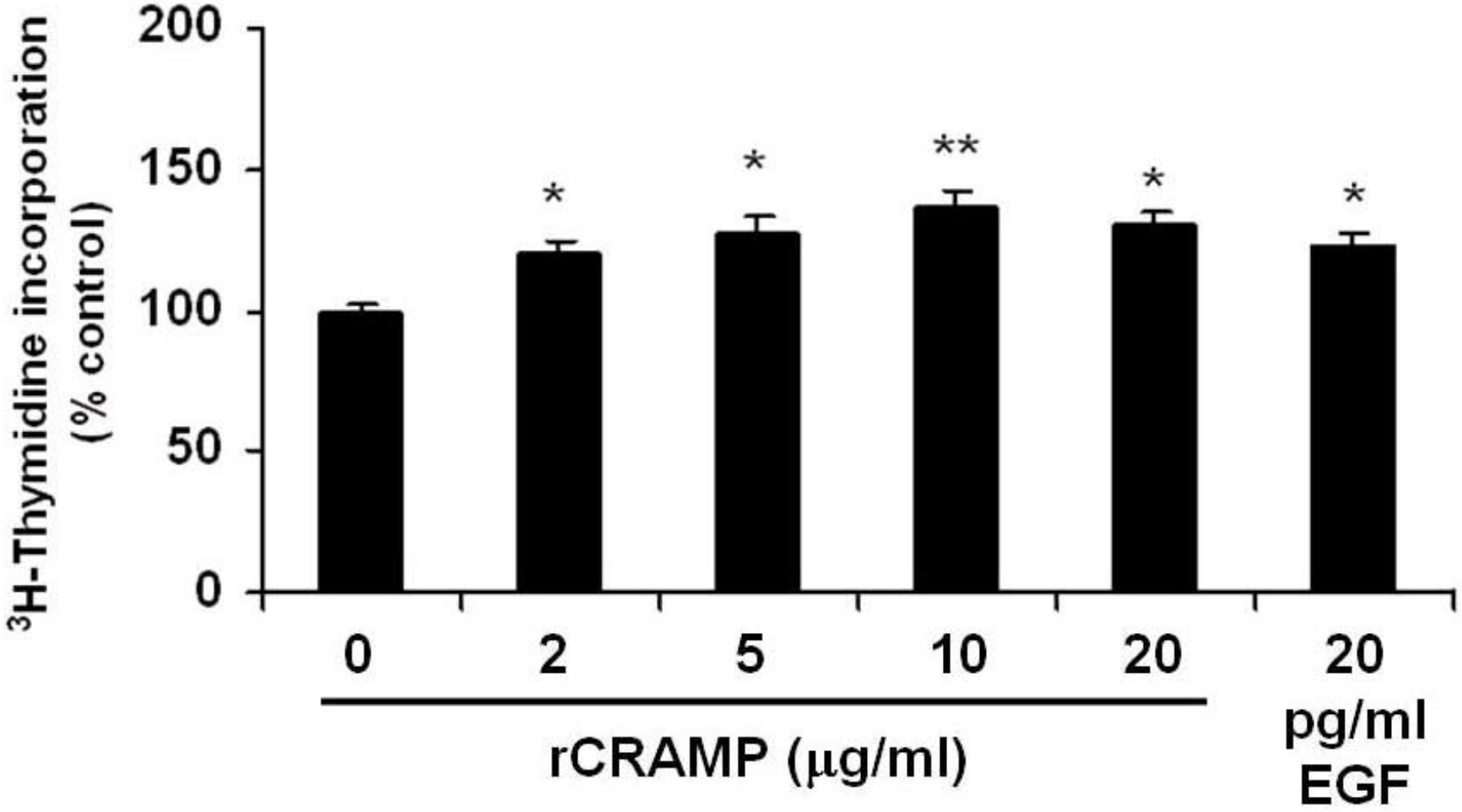


Figure 4



JPET Fast Forward. Published on May 2, 2006 as DOI: 10.1124/jpet.106.102467
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5

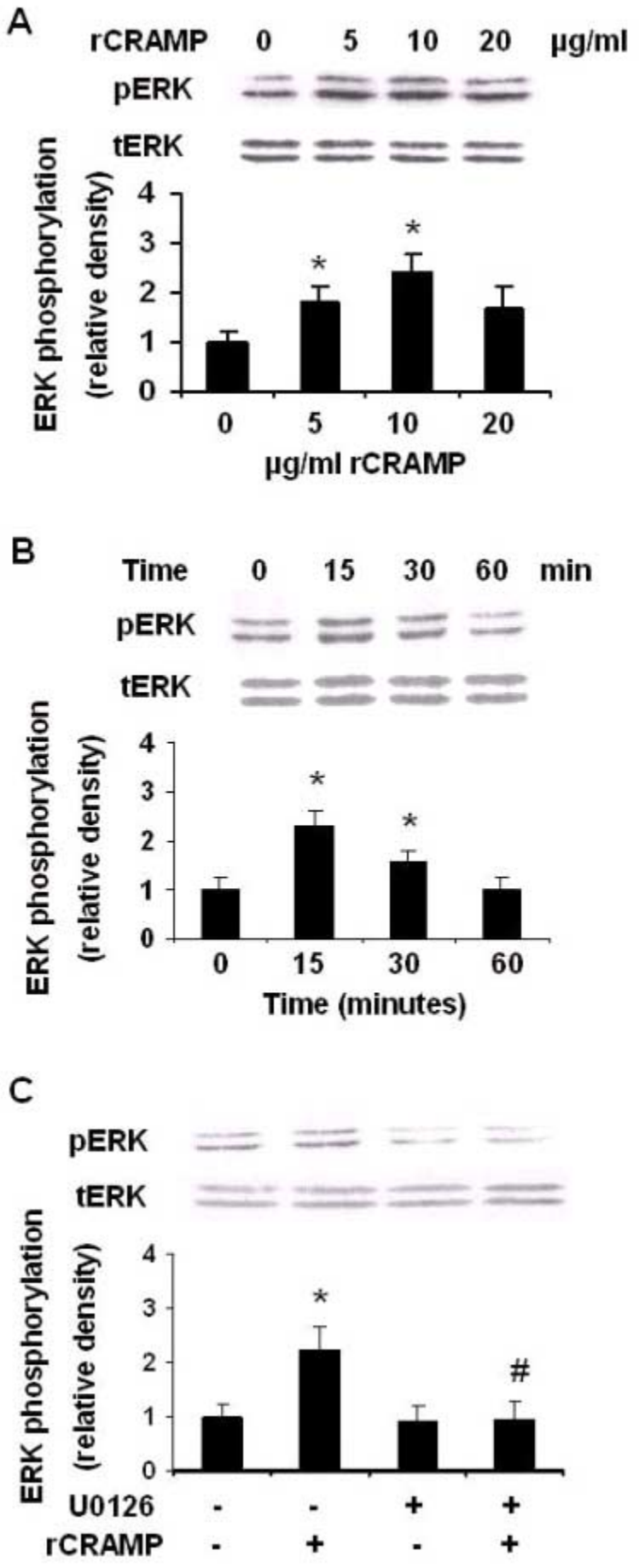
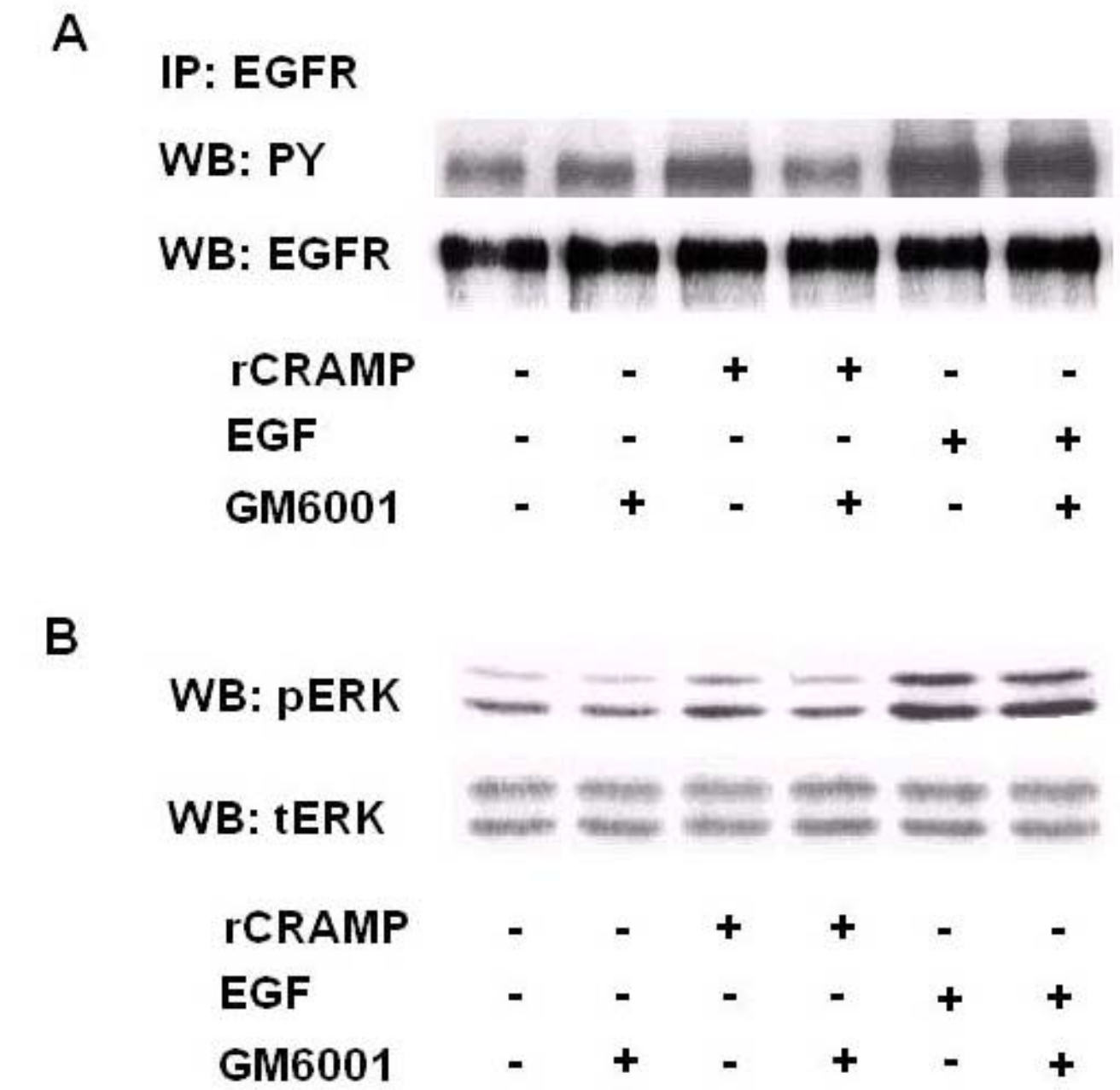


Figure 6

Figure 7



JPET Fast Forward. Published on May 2, 2006 as DOI: 10.1124/jpet.106.102467
This article has not been copyedited and formatted. The final version may differ from this version.

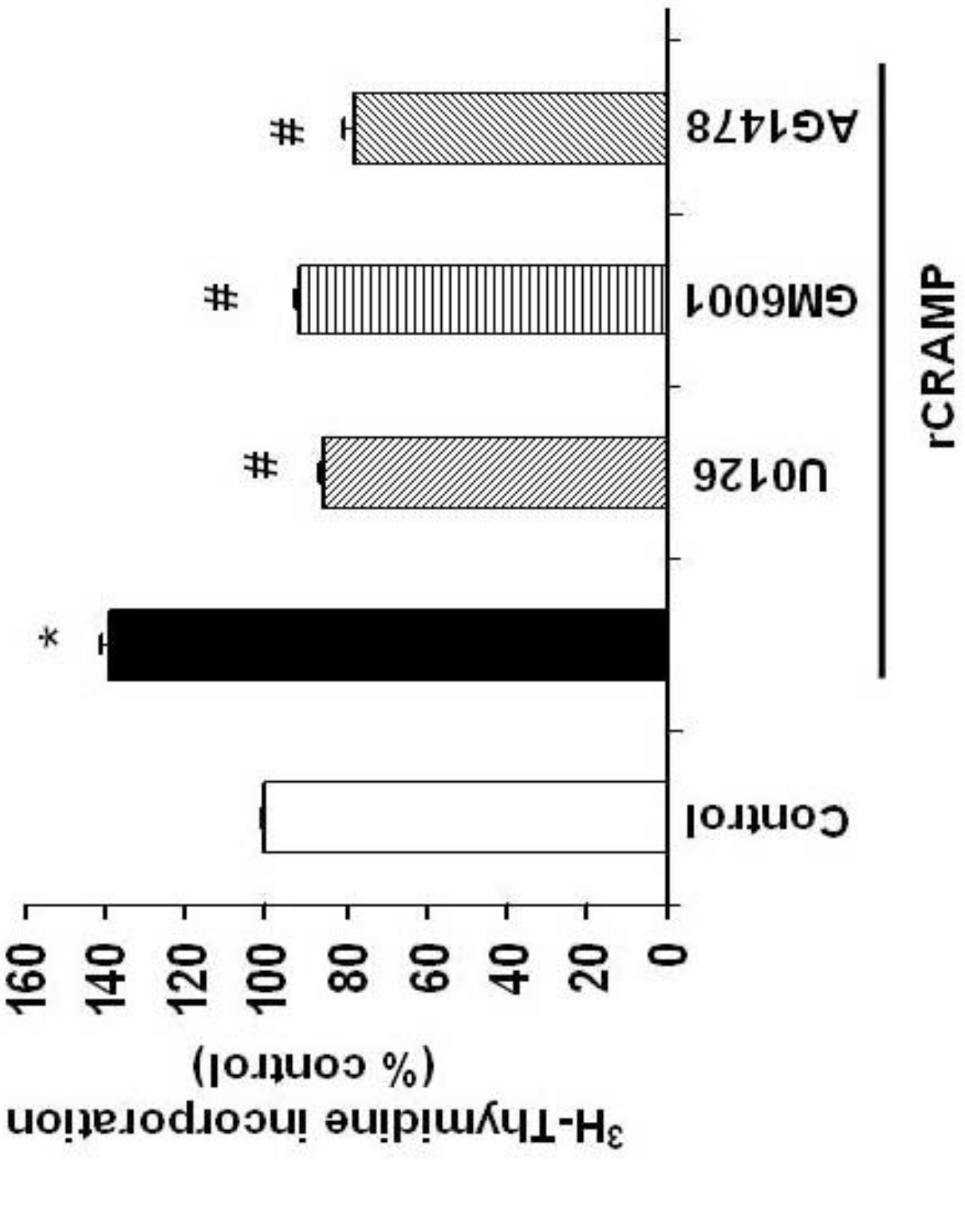


Figure 8

Figure 9

Figure 10