

Expression of functional receptor activity modifying protein 1 by airway epithelial cells with dysregulation in asthma

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Background: Epithelial cell expression of calcitonin gene-related peptide (CGRP) is a feature of provoked asthma. Receptor activity modifying protein 1 (RAMP1) and the calcitonin receptor-like receptor combine to form the CGRP1 receptor.

Objective: To determine whether functional RAMP1 is expressed by airway epithelial cells and whether there are alterations in asthma.

Methods: BEAS-2B and A549 cells lines were studied by RT-PCR, confocal microscopy, a quantitative immunofluorescence assay, and ELISA. Bronchial biopsies from normal subjects and subjects with asthma were examined by immunohistochemistry and *in situ* hybridization.

Results: Inflammatory cytokines induced CGRP release and CGRP mRNA in BEAS-2B and A549 epithelial cell lines. RAMP1 was highly expressed by resting, unstimulated BEAS-2B and A549 cells. CGRP induced internalization of RAMP1 and IL-6 production, both of which were inhibited by the CGRP antagonist, CGRP₈₋₃₇. Activation of BEAS-2B and A549 cells by inflammatory cytokines induced CGRP secretion, binding of CGRP to RAMP1, and RAMP1 internalization, which was blocked by CGRP₈₋₃₇. RAMP1 immunoreactivity and RAMP1 mRNA expression in bronchial biopsies from subjects with asthma were significantly lower than in normal subjects ($P = .002$ and $P = .007$, respectively). Inhalational challenge of atopic subjects with asthma with allergen-derived peptides produced a significant decrease in the numbers of RAMP1-positive epithelial cells in responders ($P = .027$) but not nonresponders.

Conclusion: Receptor activity modifying protein 1 was expressed both by airway epithelial cells in culture and in bronchial biopsies from normal subjects and internalized after epithelial cell activation through autocrine feedback of CGRP. There is an apparent dysregulation of RAMP1 in asthmatic

epithelium, suggesting continuous stimulation of pathways involving CGRP. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: RAMP1, CGRP, asthma, epithelial cells

Calcitonin gene-related peptide was expressed by bronchial epithelial cells of subjects with cat allergy and asthma who responded with a late-phase reaction (LAR) 6 hours after inhalation of peptides derived from Fel d 1, the major cat allergen.¹ This supported the view that edema of the airways contributed in airway narrowing in the LAR, or even ongoing asthma, and that CGRP, a potent vasodilator,² may be an important mediator in these events.

The calcitonin gene-related peptide (CGRP) receptor (sometimes referred to as the CGRP1 receptor) is a heterodimeric complex of the receptor activity modifying protein (RAMP)–1 and a family B G-protein-coupled receptor called the calcitonin receptor-like receptor (CRLR).³ The CGRP1 receptor is the best characterized, having high affinity for CGRP (~0.1–1 nmol/L). This can be competitively antagonized by CGRP₈₋₃₇.⁴ RAMPs are a family of single transmembrane domain proteins and are required to transport the CRLR to the plasma membrane.³ RAMPs are involved in the terminal glycosylation, maturation, and presentation of the appropriate receptor to the cell surface.⁵ There are 3 distinct types of RAMPs, designated RAMP1, RAMP2, and RAMP3, each encoded by a separate gene.⁶ Associations of RAMPs with either the calcitonin receptor (CR) or CRLR proteins form 6 different receptors from the calcitonin receptor family. For example, CRLR and RAMP2 form the adrenomedullin receptor designated adrenomedullin 1, and RAMP3 combines with CRLR to form the amylin receptor 3.⁷ There are other, less well defined receptors that bind CGRP with lower affinity and are weakly inhibited by CGRP₈₋₃₇. They have been historically referred to as CGRP2 receptors and include the amylin receptor 1a (a CR/RAMP1 complex) and the adrenomedullin 2 receptor (CRLR/RAMP3).⁶

Respiratory epithelial cells are known to produce and respond to a large number of cytokines and other inflammatory mediators including IL-1 β , TNF- α , and CXCL8/IL-8.^{8,9} Thus, epithelial cells can be a target for the action of cytokines released not only by inflammatory cells but also for their own mediators (ie, an autocrine effect).⁸ To investigate a possible role for CGRP in asthma, we investigated the expression and function of the CGRP receptor complex in bronchial epithelial cells in culture and in tissues of normal subjects and subjects with asthma.

METHODS

Cell culture

BEAS-2B cells were maintained in RPMI-1640 medium containing glutamine supplemented with 0.02 mol/L HEPES, 0.075% sodium

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Abbreviations used

BM:	Basement membrane
CGRP:	Calcitonin gene-related peptide
CM:	Cytokine mix
CR:	Calcitonin receptor
CRLR:	Calcitonin receptor-like receptor
LAR:	Late-phase reaction
PBS:	Phosphate-buffered saline
PBS-T:	PBS 0.05% Tween
RAMP:	Receptor activity modifying protein
SFM:	Serum-free medium
UK:	United Kingdom

bicarbonate, 10% FBS, 100 U/mL penicillin, and 50 ng/mL streptomycin and A549 cells in Dulbecco modified Eagle medium containing 400 mg/L glucose, l-glutamine, 100 mg/L sodium pyruvate supplemented with 10% FBS, 0.02 mol/L HEPES, 100 U/mL penicillin, and 50 ng/mL streptomycin. Cells were grown in a humidified incubator at 37°C and passaged at 80% confluence. Before stimulation, cells were maintained for 4 hours at 37°C in keratinocyte serum-free medium (SFM) supplemented with bovine pituitary extract and epidermal growth factor. Unless stated, materials were purchased from Gibco/Invitrogen/Life Technologies (Carlsbad, Calif). After serum starvation, SFM was removed, and conditioned SFM containing indicated concentrations of CGRP (BACHEM/ Peninsula LLC, San Carlos, Calif), CGRP₈₋₃₇ (Sigma-Aldrich, Poole, United Kingdom [UK]), and the cytokines IL-13, IL-1β, TNF-α, and GM-CSF (PeproTech EC Ltd, London, UK), added for times indicated. Supernatants for enzyme immunoassay were supplemented with a 1:1000 diluted protease inhibitor cocktail (Sigma-Aldrich) and remaining cells fixed for immunostaining or processed for RNA extraction.

Confocal microscopy

Cells were plated onto 13-mm-diameter coverslips in 24-well plates overnight at 37°C/5% CO₂. Cells were washed once in Dulbecco's phosphate-buffered saline (PBS) and fixed at room temperature for 25 minutes with 4% paraformaldehyde in PBS. After thorough washing with PBS 0.05% Tween (PBS-T), cells were permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 15 minutes. After washing 3 times in PBS-T, cells were incubated in a blocking buffer consisting of 1% BSA, 0.05% Tween, and 0.1% human AB serum in PBS, then incubated overnight in blocking buffer containing protein-specific or isotype control primary antibody overnight at 4°C. Wells were washed and incubated with an appropriate conjugated secondary antibody for 1 hour at room temperature. After thorough washing, cells were incubated for 10 minutes in 1 μmol/L TOPRO-3 (Molecular Probes/Invitrogen, Life Technologies) to stain nuclei, and mounted in Vectashield mounting solution (Vector Laboratories, Peterborough, UK). Single optical sections of cells were obtained by using a Leica TCS-NT confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). Antibodies used were as follows. As primary antibodies, 2 μg/mL rabbit polyclonal anti-human CGRP (H-48), normal rabbit IgG, goat antihuman RAMP1 (N-20), and normal goat IgG were all purchased from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). Secondary antibodies were 4 μg/mL goat antirabbit Alexa Fluor 488, donkey antigoat 546 (Molecular Probes/Invitrogen, Life Technologies), and swine antirabbit TRITC (Dako UK Ltd, Ely, UK).

Assay for internalization of RAMP1

After binding of CGRP, the receptor complex containing RAMP1 normally localized to the plasma membrane is rapidly internalized.¹⁰ Surface expression of RAMP1 protein after stimulation with agonist was determined by an immunofluorescence-based quantitative internalization assay carried out on fixed adherent cells, similar in principle to the internalization assays more commonly carried out on suspended cells by using flow cytometry. In our assay, the signal was detected by using a plate reader. Staining in the absence of

Triton permeabilization results in the signal being mainly from the surface. Differences in RAMP-1 surface expression were detected by RAMP1 immunoreactivity with and without indicated treatment. BEAS-2B cells were seeded at 1×10^4 in flat-bottomed 96-well plates and grown overnight at 37°C as described. After 4 hours in SFM, cells were treated with conditioned SFM containing indicated concentrations of CGRP (BACHEM/Peninsula LLC), CGRP₈₋₃₇ (Sigma-Aldrich), and the cytokines IL-1β, TNF-α, and GM-CSF, added for times indicated. After treatment, the supernatant was removed and the cells were washed in PBS-T and fixed in 4% paraformaldehyde for 25 minutes at room temperature. Wells were blocked for 30 minutes in blocking buffer consisting of PBS containing 1% BSA, 0.1% human A B serum, and 0.05% Tween. Cells were incubated overnight in 2 μg/mL antihuman RAMP1 (N-20) antibody (Santa Cruz Biotechnology Inc) diluted in blocking buffer or 2 μg/mL goat polyclonal isotype control (Santa Cruz Biotechnology Inc) at 4°C. Wells were washed 3 times in PBS-T and cells stained with 4 μg/mL rabbit antigoat Alexa Fluor 488 antibody (Molecular Probes/Invitrogen, Life Technologies) for 1 hour at room temperature. Cells were washed as before and incubated for 10 minutes in 300 nmol/L 4',6-diamidino-2-phenylindole, dihydrochloride in PBS at room temperature. Cells were washed once and immersed in 50 μL PBS. Fluorescence was detected on a FLUOstar Galaxy plate reader (BMG Labtech, Offenburg, Germany). Fluorescence data were normalized to 4',6-diamidino-2-phenylindole and presented as percent RAMP1 expression at baseline.

Quantitative ELISA for IL-6

BEAS-2B cells were seeded overnight in 24-well plates and allowed to reach to approximately 80% confluence in complete medium. After 4 hours of serum starvation in SFM, 0 to 100 μmol/L CGRP/SFM with or without 25 pmol/μL of the CGRP antagonist CGRP₈₋₃₇ was added to triplicate wells. After 6 hours at 37°C/5% CO₂, the supernatant was removed and IL-6 measured by ELISA (PeproTech EC Ltd). The limit of detection of the assay was approximately 32 pg/mL.

Bronchial biopsies

Bronchial biopsies were obtained from 15 subjects with mild, atopic asthma and 11 normal volunteers. The subjects' clinical features are described elsewhere¹¹⁻¹³ and in this article's Patient Clinical Details section in the Online Repository at www.jacionline.org.

Immunohistochemistry

Immunohistochemistry with the alkaline phosphatase antialkaline phosphatase method was used as previously described.¹¹ Polyclonal rabbit antihuman RAMP1 antibody (RAMP1 FL-148) was obtained from Santa Cruz Biotechnology Inc and polyclonal rabbit antimouse immunoglobulin and alkaline phosphatase antialkaline phosphatase reagents from Dako (Ely, UK). For RAMP1 immunofluorescence, tissue sections were pretreated with PBS for 30 minutes and incubated overnight at room temperature in a humidified chamber with anti-RAMP1. After extensive washing, sections were incubated with a fluorescein isothiocyanate-conjugated goat antirabbit antibody (Dako). Appropriate isotype controls were included (IgG₁, Dako). Images were acquired using a Leica TCS SP confocal microscope (Leica Microsystems).

The sections were counted blind by using an Olympus BH-2 Microscope (Olympus Corp, Lake Success, NY). The numbers of RAMP1-positive and RAMP1-negative stained epithelial cells were counted along the entire basement membrane (BM) of each section by using a squared eyepiece graticule (Olympus Corp, Lake Success, NY) and expressed as the number per unit length of BM (positive or negative cells/mm BM). RAMP1 expression by bronchial smooth muscle was quantified by using an arbitrary 1 to 5 scale as previously described.¹ On selected samples, the slides were counted in duplicate, and the coefficient of variation was <5%.

In situ hybridization

In situ hybridization was performed to identify mRNA encoding human RAMP1 (GenBank accession no. NM005855) by using fluorescein

isothiocyanate-labeled single-stranded phosphodiester DNA oligonucleotides (BIOGNOSTIK HybriProbe kit; Diagnostik, Gottingen, Germany) as previously described.^{14,15}

Statistical analyses

Results of 2 or more groups were analyzed by a Friedman repeated-measures nonparametric test, with pairs of groups analyzed by a Dunn multiple comparisons or Wilcoxon matched pairs test, as indicated. A value of $P < .05$ was considered significant. Statistical analysis and graph generation were performed by using Graph Pad Prism version 4.03 (Graph Pad Software Inc, San Diego, Calif).

The Mann-Whitney U test was used to compare baseline expression between normal volunteers and volunteers with asthma. Data were analyzed by using Graph Pad Prism (Graph Pad Software Inc).

Statistical comparisons of diluent and peptides inhalation were performed by using 2-tailed Wilcoxon signed-rank tests. Significance was accepted as $P < .05$.

RESULTS

Airway epithelial cell lines constitutively express RAMP1

Both BEAS-2B and A549 cells were found to be immunopositive for RAMP1 at baseline—that is, with no previous treatment (Fig 1). Thus, BEAS-2B and A549 cells gave constitutive RAMP1 immunoreactivity, as did airway epithelial cells obtained from bronchial biopsies from normal subjects (Fig 2). The staining of BEAS-2B and A549 cells appeared to be uniformly distributed.

CGRP induces internalization of RAMP1 and production of IL-6

To determine whether cytokine induced-CGRP secretion by bronchial epithelial cells results in autocrine signaling effects, and hence plays a functionally relevant role in epithelial activity in the inflammatory environment, the effect of cytokine-induced CGRP secretion on CGRP activity was determined by assessing internalization of RAMP1. We first established that proinflammatory cytokines induced CGRP expression (see this article's Methods and Results sections and Fig E1 in the Online Repository at www.jacionline.org). From this data and unpublished experiments, we calculated that the range of cytokine mix (CM)-induced CGRP in epithelial supernatants was between 0.013 and 263.9 nmol/L. Accordingly, we incubated BEAS-2B cells with 0, 0.01, 1, and 100 nmol/L CGRP for 1 hour and observed a dose-dependent decrease in RAMP1 surface expression, which was inhibited by the CGRP antagonist CGRP₈₋₃₇ (Fig 3, A). About a 20% decrease in RAMP1 surface expression was observed with the highest dose of CGRP, 100 nmol/L. We also observed that incubation of cells with CGRP₈₋₃₇, even in the presence of CGRP, produced an increase, over control values, in RAMP1 surface expression.

Evidence that a CGRP/CGRP receptor interaction on epithelial cells leads to release of an inflammatory mediator, and hence has relevance in airway inflammation, was obtained by demonstrating IL-6 release on incubation of BEAS-2B cells with CGRP. BEAS-2B cells were treated with 0, 0.01, 1, and 100 nmol/L CGRP alone or in combination the antagonist CGRP₈₋₃₇. CGRP induced a significant IL-6 secretion that was blocked by the CGRP₈₋₃₇. However, there was little difference in IL-6 secretion between the doses of CGRP studied, indicating that the response was maximal at a CGRP concentration of 10 pmol/L (Fig 3, B).

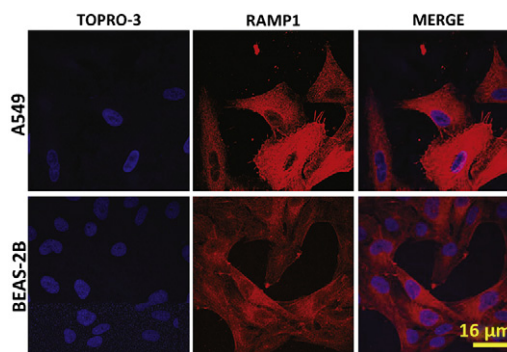


FIG 1. Airway epithelial cell lines constitutively express RAMP1. Confocal micrograph images of A549 and BEAS-2B cells labeled with a RAMP1 antibody. IgG controls are shown in this article's Fig E2 in the Online Repository at www.jacionline.org.

Cytokine-induced production of CGRP is associated with internalization of the CGRP/CGRP receptor complex

The combination of an anti-CGRP and anti-RAMP1 antibody was used to provide direct evidence of CGRP/CGRP receptor internalization and an autocrine feedback loop. Using single optical confocal microscopic sections, it was shown that there was minimal colocalization of CGRP with RAMP1 in untreated A549 cells. However, after incubation with CM, discrete intracellular aggregates of colocalization could be observed (Fig 4), indicating cytokine-mediated production of CGRP, binding of the CGRP receptor, and subsequent internalization of the complex. Similar data were obtained with BEAS-2B cells (data not shown).

Further evidence of CM-induced CGRP generation with internalization of the CGRP/CGRP receptor complex was obtained by measuring RAMP1 surface expression on BEAS-2B cells in the presence and absence of the CGRP antagonist CGRP₈₋₃₇ (Fig 5). After 24-hour incubation, CM produced a significant decrease in RAMP1 surface expression that was inhibited by CGRP₈₋₃₇. Although there was no difference in the RAMP1 internalization between 10 and 100 ng/mL CM at 24 hours, at 48 hours, 100 ng/mL CM induced greater internalization, indicating a dose-specific increase in CGRP after cytokine stimulation and subsequent receptor internalization. As noted with CGRP incubation (Fig 3, C), the antagonist, CGRP₈₋₃₇, enhanced RAMP1 surface expression compared with controls.

Asthma is associated with dysregulation of RAMP1

The expression of RAMP1 protein and RAMP1 mRNA in bronchial epithelial cells obtained from mucosal bronchial biopsies of subjects with mild asthma and normal subjects is shown in Fig 2 (upper panels). There was a highly significant decrease in both protein and mRNA when these subjects with mild, atopic asthma were compared with normal subjects ($P = .002$ and $P = .007$, respectively). There was no difference in RAMP1 expression by bronchial smooth muscle between normal subjects and subjects with asthma at baseline. However, using the arbitrary scale score previously described,¹ smooth mRNA RAMP1 expression was significantly lower in subjects with asthma (mean score, 0) than normal subjects (mean score, 2; $P = .003$; data not shown). There was also no difference in RAMP1 expression at baseline between subjects with asthma who went on to have

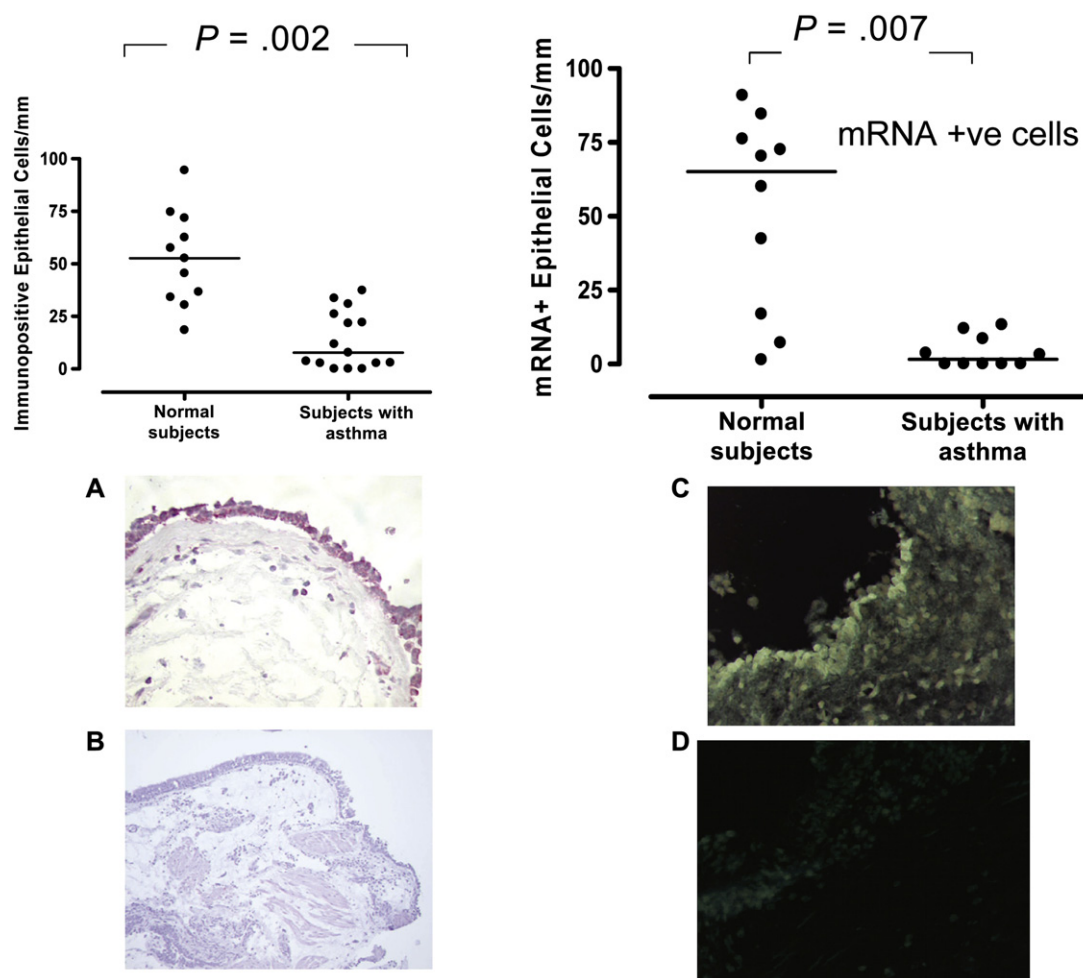


FIG 2. Epithelial RAMP1 expression in bronchial biopsies of subjects with asthma and normal subjects. **A**, RAMP1 immunostaining in a normal subject; **B**, RAMP1 immunostaining in an asthmatic; **C**, RAMP1 *in situ* hybridization in a normal subject; **D**, RAMP1 *in situ* hybridization sense control in a normal subject. Subjects with asthma (at baseline) and normal subjects were compared for the numbers of epithelial RAMP1 protein⁺ cells (top left) and RAMP1 mRNA⁺ cells (top right). *P* values were calculated using the Mann-Whitney *U* test. Photomicrograph examples of RAMP1 immunostaining and mRNA (explained in Results) are shown in the lower panels.

a dual (early-phase and late-phase reaction) compared with those who gave a single early response. Photomicrograph examples of RAMP1 immunostaining from normal subjects and subjects with asthma are shown in Fig 2. RAMP1 immunostaining of bronchial epithelium from a normal individual is shown in Fig 2, A, and from a subject with asthma in Fig 2, B. *In situ* hybridization with marked RAMP1 mRNA positivity of epithelial cells from a normal subject is illustrated in Fig 2, C, and the sense control is shown in Fig 2, D.

We also studied the bronchial epithelium of subjects with allergic asthma who had undergone bronchial inhalational challenge with allergen-derived T-cell peptide epitopes. As previously described,¹³ about 50% of these subjects develop an isolated late-phase asthmatic reaction (and are termed *responders*). We observed significantly fewer RAMP1-positive immunoreactive cells in 6-hour bronchial biopsies than prechallenge biopsies in responders ($P = .027$; Fig 6). In contrast, in individuals who did not have a late asthmatic reaction (nonresponders), there was no significant difference between prechallenge and 6 hours.

DISCUSSION

We show for the first time that human respiratory epithelial cell lines and bronchial epithelium from normal volunteers constitutively express RAMP1. We also demonstrate RAMP1 internalization after activation of lung epithelial cells either by exogenous CGRP or CGRP induced by inflammatory cytokines, and its inhibition by the CGRP receptor antagonist, CGRP₈₋₃₇. In addition, RAMP1 expression in asthma was markedly decreased, suggesting ongoing CGRP production with CGRP1 receptor internalization.

A possible role for CGRP in asthma was suggested by its identification in bronchoalveolar lavage, epithelial cells, airway inflammatory cells, and smooth muscle in allergen-peptide-induced LAR.¹ These observations are consistent with previous studies on the role of the vasculature in asthma, including increased angiogenic factors such as VEGF and angiogenin¹⁶ as well as stromal cell-derived factor 1 in asthmatic airway epithelium.¹⁷ Furthermore, increases in subepithelial vessels of the airways were observed in subjects with asthma but not patients with

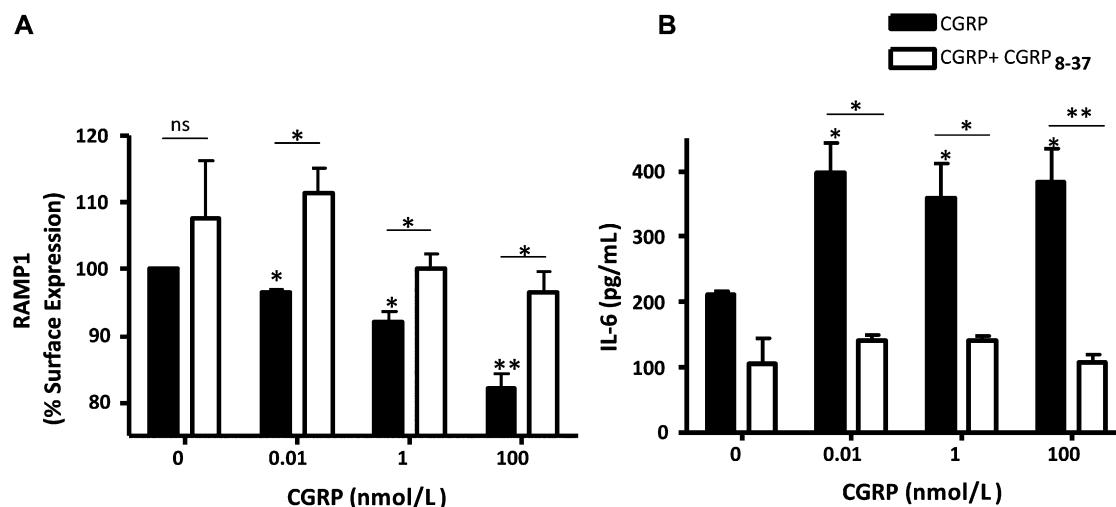


FIG 3. Exogenous CGRP induces internalization of RAMP1 (A) and production of IL-6 (B). Cells were treated with CGRP with or without CGRP₈₋₃₇. In A, bars represent mean fluorescence units as a percentage of untreated cells. In B, results are expressed as mean fluorescence units as a percentage of untreated cells, and in B as absolute values. * $P < .05$, ** $P < .01$ compared with untreated cells or treated samples as indicated. *ns*, not significant.

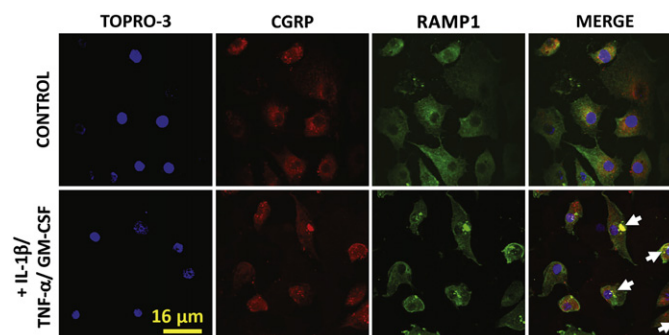


FIG 4. Endogenously secreted CGRP colocalizes with RAMP1. Confocal micrograph images showing rabbit antihuman CGRP (red) and RAMP1 (green) immunoreactivity in untreated A549 cells (control) and cells treated with 10 ng/mL proinflammatory cytokines IL-1β/TNF-α/GM-CSF. IgG controls are shown in Fig E2. Arrows indicate cells with aggregated colocalization. The TOPRO-3 and CGRP were then merged to show double-staining.

chronic obstructive pulmonary disease or normal controls.¹⁸ It also supported the observation of Aoki-Nagase et al,¹⁹ who demonstrated CGRP immunoreactivity throughout the airway epithelium and in the submucosa of mice sensitized and challenged with specific antigen. CGRP gene-disrupted mice, on the other hand, had significant attenuation of both airway hyperreactivity and CGRP expression after antigen challenge.

To confirm our previous *in vivo* findings of marked CGRP immunoreactivity of airway epithelium in bronchial biopsies from subjects with asthma taken during the LAR,¹ we first established that activated bronchial epithelial cells in culture express CGRP peptide and mRNA (Fig E1). The mixture of inflammatory cytokines produced a marked upregulation of CGRP mRNA and product when the experiments were followed over a period of 24 to 48 hours (Fig E1, A). This confirms earlier work from Li et al,²⁰ who reported that IL-1β induced CGRP in cultured rat type II alveolar epithelial cells as well as the human alveolar type II cell line, A549. These workers also reported that CGRP may work in an autocrine/paracrine mode and have an inhibitory effect because blockage of the CGRP receptor with CGRP₈₋₃₇ potentiated

IL-1β-induced release of monocyte chemoattractant protein 1 and IL-8.

Unstimulated, BEAS-2B and A549 cells and epithelial cells from human bronchial biopsies gave clear staining with a RAMP1 antibody (Figs 1 and 2). These findings were confirmed by *in situ* hybridization in which we were able to quantify RAMP1 mRNA⁺ cells in normal and asthmatic bronchial epithelium (Fig 2).

Incubation of BEAS-2B cells with increasing concentrations of CGRP led to apparent internalization of RAMP1 (Fig 3, A). The amount of internalization was modest (>20%). However, the highest concentrations of CGRP₈₋₃₇ were associated with significantly enhanced RAMP1 surface expression, indicating that there may be cycling or constitutive turnover of the RAMP1/CGRP complex under these conditions. The CGRP₈₋₃₇ sensitivity of this turnover indicates autocrine stimulation by basal secretion of CGRP. Alternatively, only a proportion of the surface RAMP1 may have been complexed with CRLR.

We used IL-6 as a marker of epithelial activation (Fig 4, B) because Veronesi et al²¹ demonstrated IL-6 release from BEAS-2B cells after stimulation with neuropeptides, including CGRP and

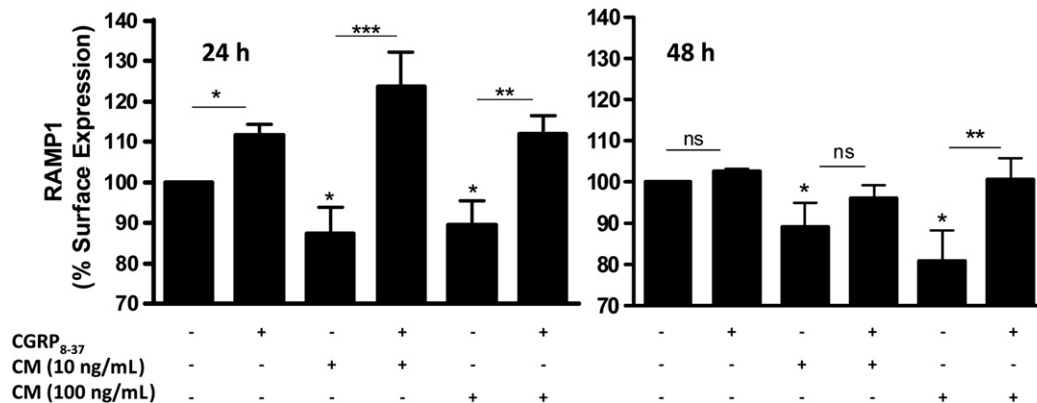


FIG 5. Endogenously secreted CGRP induces internalization of RAMP-1. BEAS-2B cells were treated with CM with or without the antagonist CGRP₈₋₃₇. Bars represent mean fluorescence units expressed as a percentage of untreated cells. Results shown are means $\pm$ SEMs of $n = 3$ independent experiments. The P values compare either CM treated with untreated cells, or CM treated cells alone with CM treated plus antagonist treated, as indicated by the isolated thin bars. * $P < .05$, ** $P < .01$, *** $P < .001$.

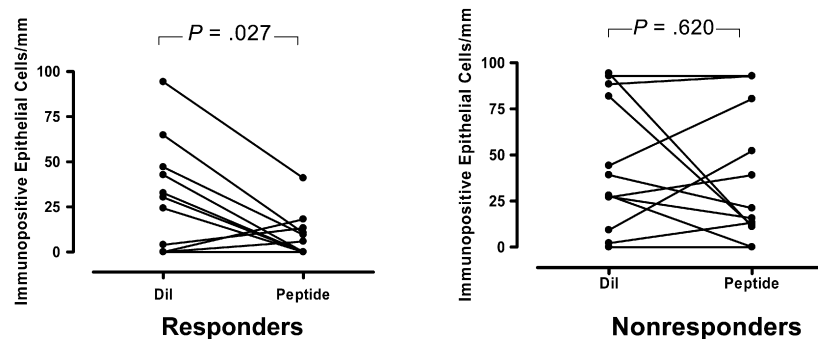


FIG 6. The effect of peptide inhalation on the numbers RAMP1⁺ immunoreactive epithelial cells in bronchial biopsies from responders and nonresponders. Statistical comparisons of diluent and peptides inhalation were performed by using 2-tailed Wilcoxon signed-rank tests. Dil, diluent.

substance P. We had previously shown that primary cultures from human bronchial epithelial cells and a tracheal epithelial cell line (9HTEo-) produced IL-6, IL-8, and GM-CSF at baseline and after stimulation with IL-1 β and TNF- α .⁹ Furthermore, Tavakoli et al²² demonstrated IL-6 release from BEAS-2B cells in response to prostaglandin E₂. There were appreciable amounts of IL-6 in unstimulated cells, as previously noted,⁸ and the increase after incubation with CGRP was modest but significant. However, at all concentrations, CGRP-induced IL-6 release was completely blocked by CGRP₈₋₃₇, suggesting that the observed effect was dependent on the CGRP1 receptor.

By 3-color confocal microscopy, we were able to show that CM induced CGRP/RAMP1 complexes in A549 cells that appeared to be both membrane-associated and internalized (Fig 4). This provided direct evidence of CGRP/RAMP1 interaction with the autocrine feedback of CGRP. We confirmed these data by showing that RAMP1 internalization after stimulation by cytokines was blocked by the CGRP1 receptor antagonist CGRP₈₋₃₇ (Fig 5). By using an immunofluorescence technique and transfected HEK 293 cells, Kuwasako et al¹⁰ demonstrated direct binding of RAMP proteins to the CRLR. CRLR was endocytosed, together with RAMPs, via clathrin-coated vesicles, and both internalized molecules were targeted to the degradative pathways.

Steiner et al²³ found that the entire single transmembrane domain of RAMP1, together with 1 hydrophilic amino acid residue at its C-terminus, is required for the formation of a fully functional CGRP/RAMP1/CRLR receptor complex. This is in contrast with RAMP2 and RAMP3, in which the C-terminal cytoplasmic tails are required for surface delivery and internalization of their respective receptor complexes.²⁴ These studies emphasize the unique specificity and function of individual RAMP proteins and suggest that our current study of RAMP1 is not reflecting activity of the structurally related adrenomedullin and amylin receptors. It is possible that a proportion of the membrane-associated molecules on the cell lines studied were heterodimeric complexes of RAMP1 with CR to form the amylin receptor 1a, which also has CGRP binding activity. In general, combinations of RAMP2 or RAMP3 with either CR or the CRLR have negligible CGRP binding activity.⁴ Thus, our data suggest that we are principally studying the CGRP₈₋₃₇-inhibitable CGRP1 receptor and that RAMP1 is a valid marker for CGRP1 receptor activity.

The marked decrease in RAMP1 protein and mRNA in subjects with asthma compared with normal subjects suggests that there may be continuous stimulation of the CGRP1 receptors by CGRP with receptor internalization (Fig 2). Rapid degradation of both the RAMP1 protein and mRNA may account for the

marked downregulation in disease. Of particular interest was the downregulation of RAMP1 in subjects with asthma who responded to inhalation of allergen-derived peptides with a late-phase reaction (Fig 6). In our previous study, we showed that these individuals, but not nonresponders, had high expression of CGRP on bronchial epithelial cells. This suggests that CGRP synthesis by epithelial cells with autocrine feedback and internalization of the CGRP1 receptor is part of the ongoing process of late asthmatic reactions and in this sense may significantly contribute to the edema and inflammation of the airway wall in this model of allergic inflammation of the airways.

In summary, we show that RAMP1 was expressed by airway epithelial cells in culture as well as bronchial biopsies from normal subjects and internalized after epithelial cell activation through autocrine feedback of CGRP. In addition, there was apparent dysregulation of RAMP1 in asthmatic epithelium, suggesting that this disease may involve continuous stimulation of pathways involving CGRP. Targeting CGRP signaling may dampen edema of the airway wall in asthma.

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Key messages

- The CGRP receptor protein, RAMP1, is expressed by airway epithelial cells and internalized after epithelial cell activation through autocrine feedback of CGRP.
- There is apparent dysregulation of RAMP1 in asthmatic epithelium, suggesting continuous stimulation by pathways involving CGRP.

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METHODS

Quantitative enzyme immunoassay

α -CGRP was measured by using a human α -CGRP high-sensitivity enzyme immunoassay kit (BACHEM/ Peninsula LLC, San Carlos, Calif). The limit of detection was approximately 8 pg/mL.

Quantitative RT-PCR

Total RNA was extracted by using Trizol reagent (Molecular Probes/Invitrogen; Life Technologies, Carlsbad, Calif) according to manufacturer's guidelines and resuspended in 50 μ L diethylpyrocarbonate-treated water, and residual DNA was removed by using a DNA free kit (Ambion; Life Technologies, Warrington, UK). One microgram of RNA was reverse-transcribed by using a high-capacity cDNA archive kit (Applied Biosystems, Life Technologies). Analysis of α -CGRP cDNA was carried out with TaqMan RT-PCR using CALCA Hs_01108255 (FAM/TAMRA) primer/probe assay. Samples were measured against a eukaryotic 18S ribosomal(r) RNA VIC/MGB probe (PrimerDesign Limited, Southampton, UK) internal positive control in a multiplex assay. Triplicate samples containing 10 μ L cDNA, 1.25 μ L CALCA, 1.25 μ L 18S and 12.5 μ L TaqMan universal mastermix were run in 96-well optical PCR plates sealed with an optical lid, and the reactions were analyzed by using an ABI7000 Automated TaqMan (Applied Biosystems, Life Technologies). The amplification cycle consisted of 50°C for 2 minutes, 94°C for 10 minutes, and 45 cycles of 94°C for 15 seconds and 60°C for 15 seconds. Data were analyzed by using v1.0 ABI Prism 7000 SDS Software (Graph Pad Software Inc, San Diego, Calif). CGRP quantitative PCR data were normalized to 18S rRNA and presented as fold induction relative to medium control at 0 hours. Unless stated, materials for quantitative RT-PCR were purchased from Applied Biosystems, Life Technologies.

RESULTS

Proinflammatory cytokines induced the expression of α -CGRP (and β -CGRP; data not shown) mRNA transcripts in BEAS-2B cells *in vitro*. In the time-course experiment shown, treatment with CM resulted in a significant time-dependent increase in α -CGRP mRNA expression after 1 hour, which increased up to 24 hours in culture, where a more than 1000-fold increase in relative mRNA expression was observed (Fig E1, A). There was also an increase in basal α -CGRP expression that peaked at 6 hours before returning to near baseline levels. In preliminary experiments, the individual cytokines alone were shown to produce only between a 2.5-fold and 10-fold increase in relative mRNA expression, and for this reason, the cytokine mixture was used in further experiments to provide maximal stimulation of epithelial cells. Similar findings to those shown in Fig E1 were obtained with A549 cells (data not shown).

Enzyme immunoassay of supernatants from BEAS-2B cells treated with CM also showed a time-dependent increase in α -CGRP. The increase continued up to 48 hours, at which time the experiment was terminated. Increases in CGRP were observed in both the CM-treated and diluent-treated samples, although cells incubated with cytokines produced significantly more CGRP than controls (Fig E1, B).

Direct evidence of cytokine-induced CGRP production by BEAS-2B cells was obtained by confocal microscopy by using a polyclonal anti-CGRP antibody specific for human CGRP that

recognizes both the α and β isoforms. There was an appreciable increase in CGRP immunoreactivity in BEAS-2B cells after treatment with CM, although there was a low background specific mean fluorescence in untreated cells. In the highly expressing cells, the stain was diffuse, whereas in lower expressing cells, CGRP was localized to the perinuclear region, possibly in association with endoplasmic reticulum (Fig E1, C). Virtually identical results were obtained with A549 cells, confirming the work of Li et al.^{E1}

PATIENT CLINICAL DETAILS

The median age of patients described in Fig 2 was 25 (range, 19-46) years, and all had an FEV₁ (% predicted) greater than 75% and a methacholine PC₂₀ less than 8 mg/mL and at least 15% reversibility to an inhaled β_2 -agonist. Volunteers sensitive to pollens were studied outside of the season and were controlled with only rescue β_2 -agonists at the time of study. They had no clinical features of infection for at least 4 weeks before starting the study and none throughout the study period. These subjects represented the study group of previous work that involved allergen inhalational challenge. Of the 15 subjects with asthma described by Kariyawasam,^{E2} 9 of these (also described by Kariyawasam^{E3}) went on to have a dual (early-phase and late-phase) asthmatic reaction, 4 had an early reaction only, and 2 were not allergen-challenged.

In the challenge study (Fig 6), bronchial biopsies were also obtained from subjects with mild asthma and cat allergy who inhaled Fel d 1-derived T-cell peptides. Full details of this patient population are also described elsewhere.^{E4} Twelve subjects developed an isolated late asthmatic reaction (greater than 20% reduction in FEV₁) to T-cell peptide. These responders (mean age, 27 years; FEV₁ [% predicted] greater than 85%; histamine PC₂₀, mean, 3.99 [range, 1.67-12.5]) completed both the control and peptide study days, as did 12 subjects who showed no clinical response (nonresponders; mean age, 28 years; FEV₁ [% predicted] greater than 86%; histamine PC₂₀, mean, 3.99, [range, 1.93-4.63]) to inhaled peptides. Both studies were approved by the Royal Brompton and Harefield Hospital Ethics Committee, and volunteers gave written informed consent. Some of the results from these studies have been presented elsewhere.^{E2-E4}

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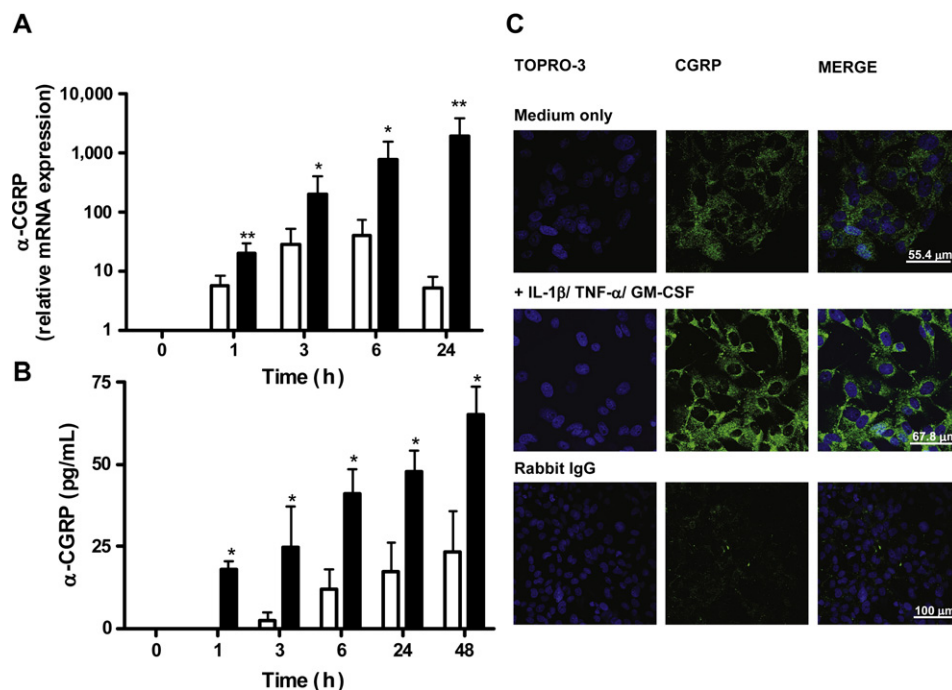


FIG E1. Proinflammatory cytokines induce CGRP mRNA expression in bronchial epithelial cell line BEAS-2B *in vitro*. **A**, α -CGRP mRNA from BEAS-2B cells cultured for 1, 3, 6, and 24 hours in supplemented SFM $\pm$ 10 ng/mL of IL-1 β /TNF- α /GM-CSF (cytokine mix). Results shown are means $\pm$ SEMs of $n = 5$ independent experiments. **B**, Cells were treated for 1 to 48 hours in SFM $\pm$ 10 ng/mL of IL-1 β /TNF- α /GM-CSF, and the α -CGRP in the supernatant was measured by enzyme immunoassay. Results shown are means $\pm$ SEMs of $n = 3$ independent experiments. *Black and open bars* represent expression of α -CGRP mRNA (**A**) or peptide product (**B**) in treated and untreated controls, respectively, relative to cells at the baseline 0 hours. Statistical significance was calculated by using the Friedman ANOVA test with a Dunn posttest. * $P < .05$ and ** $P < .01$ compared with untreated cells at same time point. **C**, BEAS-2B cells were grown overnight on coverslips and then grown in either medium alone or medium containing 10 ng/mL IL-1 β /TNF- α /GM-CSF for 24 hours. Cells were fixed and labeled with 2 μ g/mL rabbit polyclonal CGRP antibody followed by antirabbit 488 and counterstained with the nuclear stain TOPRO-3. Untreated cells were fixed and labeled similarly with an identical concentration of normal rabbit IgG. Mounted coverslips were viewed on a Leica confocal microscope (Leica Microsystems Ltd, Milton Keynes, UK). The TOPRO-3 and CGRP images were then merged to show double-staining.

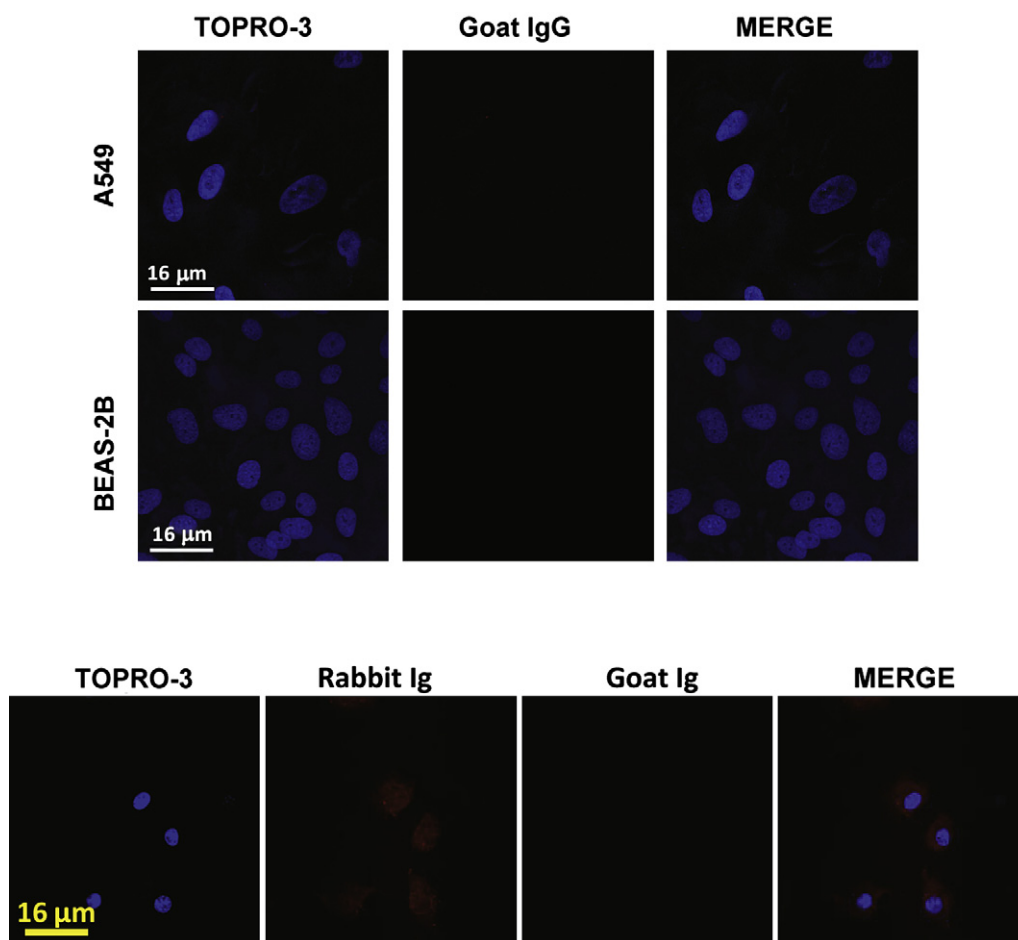


FIG E2. The IgG controls for Figs 1 and 3 are shown in Fig E2 (*upper panel and lower panel, respectively*).