Dysregulation of Reg gene expression occurs early in gastrointestinal tumorigenesis and regulates anti-apoptotic genes

Kumar S. Bishnupuri
Washington University School of Medicine in St. Louis

Qizhi Luo
Washington University School of Medicine in St. Louis

Joshua R. Korzenik
Massachusetts General Hospital

Jeffrey O. Henderson
Tabor College

Courtney W. Houchen
University of Oklahoma Health Sciences Center

See next page for additional authors

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Kumar S. Bishnupuri1
Qizhi Luo1
Joshua R. Korzenik3
Jeffrey O. Henderson4
Courtney W. Houchen5
Shrikant Anant5
Brian K. Dieckgraefe1,2

1Division of Gastroenterology and 2Siteman Cancer Center, Washington University School of Medicine, St. Louis, Missouri USA
2Division of Gastroenterology; Massachusetts General Hospital, Boston, Massachusetts USA
3Department of Biology; Tabor College; Hillsboro, Kansas USA
4Department of Internal Medicine; University of Oklahoma Health Sciences Center; Oklahoma City, Oklahoma USA

*Correspondence to: Brian K. Dieckgraefe; Washington University; Department of Internal Medicine; Division of Gastroenterology and Hepatology; St. Louis, Missouri 63110 USA; Tel.: 314.747.4659; Fax: 314.362.8959; Email: dieck@im.wustl.edu

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ABSTRACT
Expression of anti-apoptotic genes is frequently elevated in tumors, where they increase resistance to chemotherapeutic agents and predict poor patient outcomes. However, key cellular factors regulating anti-apoptotic genes in tumors remain unknown. Increased expression of the regenerating (Reg) genes is commonly observed in gastrointestinal (GI) malignancies including colorectal cancer (CRC). We therefore examined Reg gene expression and associated changes in anti-apoptotic genes in an animal model of GI tumorigenesis. Using real time RT-PCR, we measured expression of Reg genes in human colorectal adenocarcinoma specimens, colon adenocarcinoma cell lines and adenomas from multiple intestinal neoplasia (min) mice heterozygous for a germ-line mutation of the adenomatous polyposis coli (APC) gene. Expression of Reg genes is increased in human colorectal adenocarcinomas and in the intestine of APCmin/+ mice at four weeks of age, a time preceding the spontaneous second mutation in the APC gene. Individual Reg genes exhibited regional expression profiles across the GI tract in mice. Adenomas from 14-week old mice had significant increases in at least one member of the Reg gene family, most commonly Reg IV and an associated increase in expression of the anti-apoptotic gene, Bcl-2. Addition of exogenous recombinant human Reg IV to human colon adenocarcinoma cells significantly increased Bcl-2 and Bcl-xL expression and induced resistance to ionizing radiation. These results show that dysregulation of Reg genes occur early in tumorigenesis. Furthermore, increased expression of Reg genes, specifically Reg IV contribute to adenoma formation and lead to increased resistance to apoptotic cell death in CRC.

INTRODUCTION
Tumorigenesis is a multistep process involving somatic mutations or epigenetic changes affecting tumor suppressor and oncogenes.1,2 Additional genetic alterations create a permissive environment for clonal expansion of cells that are resistant to apoptosis. Advanced forms of common malignancies, such as colorectal, gastric, prostate or breast carcinoma are often associated with poor responses to adjuvant chemotherapy (CT) and/or ionizing radiation (IR).3 Apoptosis is a prominent mechanism for cell death following CT or IR.4 Accordingly, considerable attention has been given to the Bcl-2 family genes as possible regulators of intrinsic tumor resistance to therapy.5 Repressors of programmed cell death, such as Bcl-2 and Bcl-xL, decrease IR- and CT-induced apoptotic cell death in cell culture.6 However, key cellular factors that regulate expression of anti-apoptotic genes in tumors remain unknown. Defining dominant pathways responsible for modulation of apoptosis-regulating proteins would significantly enhance our understanding of tumor behavior and could broaden current strategies for therapeutic intervention.

The regenerating (Reg) genes constitute a family belonging to calcium dependent (C-type) lectin gene superfamily.7-10 The Reg family genes included six members (Reg I, Reg II, Reg IIIα, Reg IIIβ, Reg IIIδ and Reg IIIγ) in the mouse and three members (Reg Iα, Reg Iβ and Reg III) in humans.11 Human Reg IV, a novel member of the family was identified by high throughput sequencing of a library derived from patients with ulcerative colitis, constituting fourth member of the Reg gene family in human.10 A mouse homologue has also been identified constituting the seventh member in mouse. Expression of Reg genes is increased following injury, supporting a potential role in tissue repair and regeneration.12,13 Expression of Reg proteins by colorectal, gastric, and pancreatic adenocarcinomas have recently been shown to have an adverse association with patient survival.14-16 Reg IV was among several genes with increased expression in cancer cell lines selected for increased in vitro resistance to the chemotherapeutic agent, 5-FU.6 Reg IV expression was
associated with intestinal differentiation in gastric adenocarcinoma and highly elevated in colorectal cancer (CRC). Reg IV has also been identified as a promising marker of hormone refractory metastatic prostate cancer. Recently we observed the mitogenic effect of Reg IV protein, when added to the cultures of human colon adenocarcinoma cell lines with subsequent changes in expression of genes associated with altered apoptosis and metastasis. This supports the hypothesis that Reg gene products are responsible for altered apoptosis associated with a more aggressive tumor phenotype. Here we examined the expression of individual Reg genes in human colorectal adenocarcinoma specimens and adenomas from multiple intestinal neoplasia (Min) mice heterozygous for a germ-line nonsense mutation of the adenomatous polyposis coli (APC) gene. These animals spontaneously develop multiple polyps in the small and large intestine at 10–12 weeks of age following spontaneous second mutation in the APC gene. This study shows specific regional expression profiles of Reg genes along the cranio-caudal axis of the GI tract. Our results identify aberrant expression of the Reg genes as one of the earliest events in gastrointestinal tumorigenesis. Reg IV was specifically upregulated at the time of adenoma formation and contributed to the increased resistance to apoptotic cell death.

**MATERIALS AND METHODS**

**Cell lines and culture.** HCT116 and HT29 colon adenocarcinoma cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (Cambrex, Walkersville, MD) containing 10% heat inactivated fetal bovine serum (Sigma, St. Louis, MO). Cells were placed in serum-free media overnight prior to treatment with endotoxin-free recombinant human recombinant serum (Sigma, St. Louis, MO). Cells were incubated in serum-free media overnight prior to treatment with endotoxin-free recombinant human recombinant serum (Sigma, St. Louis, MO). Cells were placed in serum-free media overnight prior to treatment with endotoxin-free recombinant human recombinant serum (Sigma, St. Louis, MO). Cells were placed in serum-free media overnight prior to treatment with endotoxin-free recombinant human recombinant serum (Sigma, St. Louis, MO). Cells were placed in serum-free media overnight prior to treatment with endotoxin-free recombinant human recombinant serum (Sigma, St. Louis, MO).

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species*</th>
<th>Primer</th>
<th>Probe &amp; dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>M</td>
<td>GGCAAATTCACCGCACAGT AGATGGTGATGGGCTTCCC</td>
<td>JOE/AGGCCGAGAATGGGAAGCTTGCAC/BHQ</td>
</tr>
<tr>
<td>β Actin</td>
<td>H, M</td>
<td>ATCATGTCCTCTCGAGGCG (F) GCTGATCCACATCTGCGAA (R)</td>
<td>SYBR</td>
</tr>
<tr>
<td>Reg I</td>
<td>M</td>
<td>CATCTCGCTCTCATGCTGAT (F) GCAGATGGCAGCTTCTTCCA (R)</td>
<td>TET/CCTGCTCAAGGAGGCTGAGG/BHQ</td>
</tr>
<tr>
<td>Reg la</td>
<td>H</td>
<td>TGAATCTGCTGCTGTGACCCA (F) CCTCCTGGACGCTGATCC (R)</td>
<td>SYBR</td>
</tr>
<tr>
<td>Reg lb</td>
<td>H</td>
<td>TCTAGAGGAGGCCAG (F) GGCCTGCGGCCAGCTGAT (R)</td>
<td>SYBR</td>
</tr>
<tr>
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<td>M</td>
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</tr>
<tr>
<td>Reg III</td>
<td>H</td>
<td>GTAACAGCTACATCGTCTGGA (F) CTCACACCCGACCTGTGGA (R)</td>
<td>SYBR</td>
</tr>
<tr>
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<td>M</td>
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</tr>
<tr>
<td>Reg IIIb</td>
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<td>TET/TCCTGAGGACTGAGCTTCC/BHQ</td>
</tr>
<tr>
<td>Reg IIIγ</td>
<td>M</td>
<td>GGTGCGCTGATGGT (F) AGCTGAGTGTCTGGAGAAGAC (R)</td>
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</tr>
<tr>
<td>Reg IV</td>
<td>M</td>
<td>GAGTACAGCTGCGGCAG (F) CACACTCTGCGGCTT (R)</td>
<td>TET/TCCTGAGGACTGAGCTTCC/BHQ</td>
</tr>
<tr>
<td>Reg IV</td>
<td>H</td>
<td>TGGATCTGCTGCGGCAG (F) CACACTCTGCGGCTT (R)</td>
<td>FAM/TCTCTGGAGTGTCTGCGATCCA/BHQ</td>
</tr>
<tr>
<td>Bcl 2</td>
<td>M</td>
<td>TGGATCCAGAGAGAGAGAG (F) CAAAGCGAGGTGCATGCTG (R)</td>
<td>SYBR</td>
</tr>
<tr>
<td>Bcl 2</td>
<td>H</td>
<td>CGTGCCTTCGGCTCTT (F) AGCTGAGTGTCTGGAGAAGAC (R)</td>
<td>SYBR</td>
</tr>
<tr>
<td>Bcl xL</td>
<td>H</td>
<td>CCCATGCTCCTCCTG (F) TAAGCTGCGGATCCCTG (R)</td>
<td>SYBR</td>
</tr>
</tbody>
</table>

*H, human; M, murine.*
Reg IV is most upregulated gene of Reg family in human colorectal adenocarcinomas. In order to determine Reg gene expression in gastrointestinal tumors, we measured the expression of human Reg genes in 14 colorectal adenocarcinoma resection specimens and paired normal mucosa. mRNA expression of individual Reg genes was determined by real time RT-PCR and normalized to β-actin expression. Absolute mRNA level of individual Reg gene was determined based on titrated standard curve using specific primer sets and probes. Reg IV was the dominant member of the Reg gene family expressed in normal mucosa (mRNA expression: 6.1 ± 4.5, 0.45 ± 0.2, 0.05 ± 0.03, and 27.2 ± 9.3 of Reg Iα, Reg Iβ, Reg III and Reg IV respectively) (Fig. 1A). While colorectal adenocarcinoma specimens exhibited increased expression of all Reg genes, Reg IV, and to a lesser extent Reg Iα constituted the dominant members of the Reg gene family expressed by colorectal adenocarcinomas (mRNA expression: 492 ± 304, 56 ± 33, 8 ± 6, and 1456 ± 676 of Reg Iα, Reg Iβ, Reg III and Reg IV respectively) (Fig. 1A). Individual resected tumors showed highly unique patterns of Reg gene expression. For example, specimen 3 had increased Reg IV expression only; however, specimen 2 had increases in expression of all members of the Reg gene family including Reg IV. 100%, 86%, 86%, and 71% of colorectal adenocarcinoma specimens demonstrated increased Reg IV, III, Iα, and Iβ expression respectively, greater than 1.5-fold relative to adjacent normal mucosa (Fig. 1B). No clear correlation between levels of Reg gene expression and a particular histopathology or tumor stage was observed (data not shown). Consistent with increased Reg IV mRNA, colorectal adenocarcinomas showed prominent expression of Reg IV protein by immunohistochemistry (Fig. 1C).

Expression of Reg genes is dysregulated early in tumorigenesis. We first characterized the expression of the Reg gene family in the normal murine GI tract. Total RNA was isolated from individual segments of the GI tract, extending from the stomach to the colon of 14-week old mice. Reg gene expression was then determined by using Taqman probe and primer sets specific for each of the 7 Reg genes in the murine genome. Individual Reg genes displayed one of three embryologically-derived expression patterns across the cranio-caudal axis of the adult GI tract (Fig. 2A). Reg I, II and IIIβ had maximal expression in the foregut-derived stomach and duodenum; Reg IIIα, IIIβ, and IIIγ in midgut-derived small bowel extending from the jejunum to the ileum, whereas Reg IV was unique with prominent expression in the cecum and colon. We next utilized APCmin/+ mice to determine at what step expression of individual Reg genes becomes dysregulated during tumorigenesis. We analyzed regional expression of different Reg genes in the intestines of four-week old APCmin/+ mice and wild-type (APC+/+) littermate controls. Four-week old wild-type APC+/+ mice had regional expression patterns mirroring that shown in 14-week normal adults (data not shown). Compared to wild-type APC+/+ mice, different intestinal segments of four-week old APCmin/+ mice showed significant increases in expression of all Reg genes, except Reg IV (Fig. 2B). Intestinal regions associated with the greatest predisposition for adenomatous polyp formation had increases of greater than two-fold in Reg I, II, IIIβ and IIIγ expression. Furthermore, segmental increases were seen in both Reg members expressed normally at that site (e.g., Reg IIIβ in the ileum)
as well as Reg genes not expressed at that location (e.g., Reg II and III). Further, to determine changes in Reg gene expression associated with adenoma development, visible adenomas and adjacent normal mucosa were microdissected from 14-week \( \text{APC}^{\text{min/+}} \) mice. Cox-2 expression was used as a marker for successful adenoma isolation. Reg IV represented the most commonly increased member of the family in 19 individual adenomas when compared to their adjacent normal mucosa (≥1.5 fold in 72% of adenomas) (Fig. 3). Similar expression profiles of individual Reg genes were also observed in each adenoma examined, mirroring human colorectal adenocarcinoma specimens.

**Reg IV regulates anti-apoptotic genes.** Increased Bcl-2 and Bcl-x expression is a frequent occurrence in colon adenocarcinomas and is predictive of poor prognosis. To show an upregulation of Bcl-2 in \( \text{APC}^{\text{min/+}} \) mice, we examined Bcl-2 expression in adenomas by immunohistochemistry (Fig. 4A). In 14-week old wild-type \( \text{APC}^{+/+} \) mice (WT), staining was largely confined to the lamina propria immune cell populations (left panel). Epithelial staining in macroscopically normal appearing mucosa from \( \text{APC}^{\text{min/+}} \) mice was restricted to microadenomas (middle panel). However, the epithelium in microdissected gross adenomas from \( \text{APC}^{\text{min/+}} \) mice (Ade) was globally stained for Bcl-2 (right panel). Bcl-2 mRNA expression was also increased in adenomas and closely paralleled changes in Bcl-2 protein staining (Fig. 4B). The pronounced increase in Bcl-2 mRNA occurred coincides with the development of macroscopic adenomas. In addition, adenomas from 14-week \( \text{APC}^{\text{min/+}} \) mice with increased Reg IV expression generally demonstrated an associated increase in Bcl-2 mRNA (Fig. 4C). To establish a causative association between Reg IV and anti-apoptotic genes, 100 nM rhR4 was added to cultures of human colon adenocarcinoma cell lines (Fig. 5). Bcl-2 and Bcl-x mRNA expression in HCT116 (left panel) and HT29 (right panel) cells were determined by real time RT-PCR analysis. Bcl-2 expression...
Reg Gene Expression in Tumorigenesis

was increased significantly following rhR4-treatment for 2 h in HCT116 and 1 h in HT29 cells (Fig. 5A). In addition, significant increases in Bcl-xL expression were observed, when rhR4 was added to the culture medium of HCT116 cells for 2 h and HT29 cells for 3 h (Fig. 5A). To determine if the increases in Bcl-2 and Bcl-xL mRNA were associated with changes in protein expression, western blotting was performed on cell lysates isolated following rhR4 treatment. Corresponding increases in Bcl-2 and Bcl-xL protein were observed in HCT116 and HT29 cells (Fig. 5B). In response to rhR4-treatments, increases in Bcl-2 expression occurred earlier than that of Bcl-xL. These data show that exogenous Reg IV regulates expression of Bcl-2 and Bcl-xL genes and supported our previous finding of increased expression of anti-apoptotic genes in response to elevated level of Reg IV protein in colorectal adenocarcinoma cells.21

Reg IV treatment induces cell survival against radiation-induced apoptosis. Repressors of programmed cell death may directly increase resistance to therapy-induced cell death. We therefore investigated a possible protective role of Reg IV in human colon adenocarcinoma cells using an in vitro radiation-survival colony assay (Fig. 6). HCT116 and HT29 cells grown on culture plates containing media with or without 100nM of rhR4 were exposed to 4 Gy IR. The microscopically visible colonies in this model are reflective of a single surviving and proliferating cell. Exogenous Reg IV treatment significantly increased the number of colonies. Following 4 Gy IR, colony counts increased from 34.8 ± 5.1 to 50.4 ± 3.5 (45% increase, p < 0.05) in HCT116 (left panel) and

Figure 4. Bcl-2 expression is increased in microdissected adenomas from 14-week APCmin/+ mice. (A) Representative sections demonstrate normal ileum from 14-week old wild-type APC+/+ mice (normal) showing prominent Bcl-2 staining (arrowheads) in lamina propria immune cell populations but not in epithelial cells (left panel), ileac microadenoma from 14-week APCmin/+ mice (microadenoma) showing strong Bcl-2 staining of the adenoma (arrowheads) but in the normal surrounding epithelium (middle panel), and ileac gross adenomas from 14-week APCmin/+ mice (adenoma) showing global staining of epithelium cells (right panel). (B) Relative to normal mucosa from wild-type APC+/+ mice (WT), Bcl-2 mRNA expression is increased in histologically normal mucosa (µAde) and adenomas (Ade) from 14-week APCmin/+ mice (n = 5). A pronounced increase in Bcl-2 mRNA occurs coincident with the development of frank adenomas. (C) Relative to histologically normal mucosa, fold increases in Reg IV and Bcl-2 expression in adenomas from 14-week APCmin/+ mice were used to determine correlation between these two genes. Adenomas from APCmin/+ mice with increased Reg IV expression demonstrated an associated increase in Bcl-2 mRNA (Correlation coefficient, R² = 0.716).

Figure 5. Reg IV treatments increase anti-apoptotic genes, Bcl-2 and Bcl-xL in human colon adenocarcinoma cells. (A) HCT116 and HT29 human colon adenocarcinoma cell monolayer were treated for various times (h) with 100 nM rhR4. SYBR green nucleic acid dye and specific primer sets were used to determine mRNA expression of Bcl-2 and Bcl-xL. Addition of rhR4 to HCT116 cells (left panel) and HT29 cells (right panel) significantly increased Bcl-2 and Bcl-xL gene expression (*p < 0.05). B) Western blot showing Bcl-2 and Bcl-xL protein expression at various times (h) following addition of 100 nM rhR4 to HCT116 (left panel) and HT29 cells (right panel). Lower panel indicates the intensity of bands by densitometry scanning. Treatments of rhR4 to HCT116 and HT29 cells led to increased expression of Bcl-2 and Bcl-xL proteins and demonstrated parallel changes to their respective mRNA expression.
It is likely that a variety of cellular pathways may contribute to decreased apoptosis at specific stages of tumorigenesis. This may also be required for the development of a malignant phenotype. The $APC_{min/+}$ mouse model was chosen, because it mimics the developmental process of human GI tumorigenesis. These mice spontaneously develop multiple adenomas in small and large intestine at around 10–12 weeks of age following a second spontaneous mutation in the $APC$ gene. Compared to wild-type littermate controls ($APC^{+/+}$), four-week old $APC_{min/+}$ mice already had significant increases in expression of Reg genes, preceding the second spontaneous mutation in the $APC$ gene. However, increased Reg IV expression was not detected prior to the polyp formation or adenomatous changes by histology. Significant increases in Reg IV expression were noted in a series of adenomas microdissected from 14-week old $APC_{min/+}$ mice. This data is first of its kind to demonstrate that the Reg IV might have a potential role during adenoma formation following second spontaneous mutation in $APC$ gene. These data also demonstrated that adenoma formation was associated with increased expression of at least one Reg gene, mirroring the results observed in human colorectal adenocarcinomas. Our results in the $APC_{min/+}$ mouse model are in agreement with findings of a 2–3 fold increase in Reg IV expression in flat colonic mucosa containing microscopic adenomatous changes in three patients with familial adenomatous polyposis (data not shown).

Increases in Reg IV expression in adenomas of 14-week old $APC_{min/+}$ mice had an associated increase in Bcl-2 expression. Human colorectal adenocarcinoma specimens with increased Reg IV expression exhibited increased expression of Bcl-2 and Bcl-2 mRNA (data not shown). A causative role for Reg IV in regulation of the Bcl-2 family genes has been confirmed with increased expression of Bcl-2 and Bcl-2 at mRNA and protein levels following addition of rhR4 to culture media of human colon adenocarcinoma cell lines. This result supported our previous finding of increased expression of anti-apoptotic genes via Reg IV-mediated EGFR/Akt signaling cascades. Furthermore, addition of rhR4 to human colon adenocarcinoma cells led to significantly greater resistance to cell death following IR. Functional blocking of Reg IV protein increased cell susceptibility to IR-induced death (data not shown). Collectively, these data suggest that up-regulation of Reg IV and corresponding changes in cellular predisposition to apoptosis may play a requisite early role in adenoma formation.

DISCUSSION

Most adenocarcinomas are relatively resistant to CT and IR. Efforts to overcome this resistance by increasing concentration of cytotoxic drugs or dosage of irradiation has been failed to significantly improve the therapeutic response. Apoptosis is a prominent mechanism for death of cancer cells following CT or IR. Novel cancer treatment strategies targeting the genes that promote apoptosis or blocking factors that inhibit apoptosis may prove advantageous in the treatment of many malignancies. The expression of the Reg gene family is increased in common malignancies including colorectal, gastric, hepatocellular and pancreatic adenocarcinomas and may have an adverse association with patient outcomes or survival. We observed increased expression of all genes of Reg family in a series of human colorectal adenocarcinoma specimens. Reg IV was the most upregulated member of the family in these specimens. This was in accordance with previously reported data showing elevated expression of Reg IV in colorectal carcinomas. The multistep tumorigenesis model proposed by Vogelstein and Fearon describes step-wise transformation from the benign polyp to the malignant phenotype of colorectal cancer. A similar multistep process was also observed in a hepatocarcinogenesis model in rats. In each of these models, it appears that tumorigenesis is associated with an increased cell proliferation and an associated decrease in apoptotic cell death. It is likely that a variety of cellular pathways may contribute to decreased apoptosis in these stages of tumorigenesis.

Figure 6. In vitro radiation-survival colony assay showing decreases in radiation-induced apoptosis following rhR4 treatment in human colon adenocarcinoma cells. HCT116 (left panel) and HT29 cells (right panel) were plated at $10^4$ cells/25 cm$^2$ flask and incubated overnight to allow cell adherence. Cultures were then treated with complete media with or without 100 nM rhR4. After 18 hours cells were subjected to either 0 or 4 Gy IR and allowed to grow until the development of visible colonies. Colonies counted reflect survival and proliferation of individual cells (lower panel). Addition of rhR4 to HCT116 and HT29 cells significantly increased the number of surviving cells following 4 Gy IR (A and C, $p < 0.05$, control vs. 4 Gy IR; B and D, $p < 0.05$, 4 Gy IR vs. 4 Gy IR + rhR4 with HCT116 and HT29 cells respectively).

$36.8 \pm 3.5$ to $60.3 \pm 5.7$ (64% increase, $p<0.005$) in HT29 cells (right panel). In the absence of IR, rhR4 treatment did not result in a difference in the number of colonies. This data indicates that Reg IV promotes tumor cell survival following a potent apoptotic stimulus.
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