Bombesin-like peptide receptor gene expression, regulation, and function in fetal murine lung

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1Department of Pathology, Children’s and Brigham and Women’s Hospitals and Harvard Medical School, and 2Department of Medicine, Children’s Hospital, Boston, Massachusetts 02115; 3Department of Pediatrics, Harbor-UCLA Research and Educational Institute, Torrance, California 90502; 4Department of Degenerative Neurological Diseases, National Institute of Neuroscience, Tokyo 187-8502; and 5Japan Society for the Promotion of Science, Tokyo 102-8471, Japan

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Shan, Lin, Rodica L. Emanuel, Denise Dewald, John S. Torday, Nithiananthan Asokanathan, Keiji Wada, Etsuko Wada, and Mary E. Sunday. Bombesin-like peptide receptor gene expression, regulation, and function in fetal murine lung. Am J Physiol Lung Cell Mol Physiol 286: L165–L173, 2004. First published September 5, 2003; 10.1152/ajplung.00436.2002.—Bombesin-like peptide (BLP) immunoreactivity occurs at high levels in fetal lung. Previous studies showed that bombesin promotes fetal lung development. To test the hypothesis that such effects are mediated by known mammalian bombesin receptors [gastrin-releasing peptide (GRP)/bombesin-prefering receptor (GRPR), neuromedin B (NMB) receptor (NMBR), and the orphan bombesin receptor subtype-3 (BRS-3)], we analyzed the ontogeny of GRPR, NMBR, and BRS-3 gene expression in mouse lung. We examined the regulation of these three genes by dexamethasone and bombesin, which modulate lung development. Using incorporation of [3H]thymidine and [3H]choline, we then assessed whether GRP, NMB, and Leu*-phyllollitorin modulate lung growth and maturation in fetal lung explants. GRPR gene expression was detected predominantly in utero, whereas NMBR and BRS-3 genes were expressed from embryonic days 13–16 and on multiple postnatal days. All three mRNAs are present in airway epithelium and mesenchymal cells but occur in different relative patterns. These genes were regulated differently. Dexamethasone and bombesin increased GRPR mRNA, bombesin downregulated NMBR, and neither agent affected BRS-3. GRP increased incorporation of [3H]thymidine and [3H]choline in explants, whereas NMB induced cell proliferation and Leu*-phyllollitorin yielded variable results. Cumulative data suggest the involvement of multiple BLP receptors, including novel molecules, and argue against simple functional redundancy within this gene family during lung development.

Bombesin is a 14-amino acid peptide first isolated from frog skin by Anastasi et al. in 1971 (3). With the use of antibodies to amphibian bombesin, bombesin-like peptide (BLP) immunoreactivity was identified in mammalian brain, gut, and lung. The highest BLP levels were observed in human fetal lung, localized to the pulmonary neuroendocrine cells (16). One major pulmonary BLP was subsequently determined to be “gastrin-releasing peptide” (GRP), a 27-amino acid homolog of frog bombesin (Table 1). GRP and bombesin share a highly conserved seven-amino acid COOH-terminal sequence (30), which is required for immunogenicity and for high-affinity binding to the bombesin/GRP-prefering receptor. Consequently, GRP and bombesin have essentially identical physiological effects, acting as growth factors and neuroregulatory peptides (30).

Other BLPs include the amphibian phyllolitorins (PLs), which are immunologically and functionally similar to BLP (20, 22). Identified as potent smooth muscle constrictor peptides in rodents (10), putative mammalian PLs became of major interest when their potential clinical relevance emerged. BLP immunoreactivity in bronchoalveolar lavage fluid from asymptomatic human smokers was found to have an amino acid content most consistent with a PL-like peptide (1). However, this mammalian homolog has yet to be identified. PLs elicit the same panel of effects as BLP, including cell proliferation (19, 22).

Several major developmental effects of BLP have been defined. In embryonic mouse lung buds, BLP induces branching morphogenesis (19). In mice and baboons, BLP promotes widespread cell proliferation and differentiation of type II pneumocytes and neuroendocrine cells (9, 29), as well as surfactant phospholipid secretion (4). The effects of BLP can be blocked by BLP receptor-specific antagonists, which can inhibit type II cell differentiation in fetal primate lung explants (9, 35).

Three mammalian BLP receptors have been cloned (6, 23): GRP/bombesin-prefering receptors (GRPR) (7, 27), neuromedin B (NMB) receptors (NMBR) (33), and the orphan bombesin receptor subtype 3 (BRS-3) (12, 15). GRPR and BRS-3 are expressed in primate fetal lung (9). These are seven-transmembrane-spanning, G protein-coupled receptors primarily leading to PKC-dependent signal transduction with inositol trisphosphate generation (13). BLP treatment of 3T3 embryonic fibroblasts leads to tyrosine phosphorylation of focal adhesion kinase (p125 FAK ) preceding MAP kinase activation and cell proliferation (26, 37). By in situ hybridization, GRPR mRNA is detected in the mesenchyme around developing airways and blood vessels in mice and in epithelial cells in rodents, humans, and nonhuman primates (9, 19, 21, 31, 34). The effect of BLP on surfactant phospholipid synthesis by purified type II cells requires coculture with fetal pulmonary fibroblasts (8). GRPR mRNA levels in fetal lung mesenchyme peak during the canalicular phase, preceding type II pneumocyte differentiation (19, 31, 34). Subsequently, GRPR and GRP...
levels fall to low or undetectable levels during normal alveolarization, especially after birth (19, 29).

BLPs have been demonstrated to play a significant role in normal fetal lung growth and maturation (28). These effects include the promotion of branching morphogenesis in embryonic lung buds, which is optimally induced by an amphibian bombesin-related peptide, Leu²-PL (L8PL). In fetal mouse lung explants, bombesin was shown to promote fetal lung development, but no other BLP was tested in mice. In mouse lung explants, bombesin was shown to promote fetal lung buds, which is optimally induced by an amphibian bombesin-related peptide, Leu²-PL (L8PL). In fetal mouse lung explants, bombesin was shown to promote fetal lung development, but no other BLP was tested in mice. In fetal baboon lung explants, similar effects were observed using bombesin, GRP, or L8PL, but no significant effects occurred with NMB (9).

In the present study, we tested the hypothesis that different BLPs regulate fetal lung development via one or more of the known mammalian BLP receptors: GRPR, NMBR, and/or BRS-3. 1) We analyzed the ontogeny of BLP and BLP receptor gene expression and the localization of these mRNAs in developing murine lung. 2) We assessed the regulation of BLP receptor gene expression by agents known to modulate lung morphogenesis. 3) Using dose-response analyses to begin to address potential receptor specificities, we determined the effects of several previously untested BLPs on type II cell differentiation and cell proliferation in normal fetal mouse lung explants. Cumulatively, our observations support a direct role for multiple BLP receptors in murine fetal lung development.

MATERIALS AND METHODS

Animals

Timed-pregnant Swiss-Webster mice were obtained from Taconic Laboratories (Germantown, NY) at gestational day 7. We strictly adhered to the National Research Council Guide for the Care and Use of Laboratory Animals throughout all phases of the study. The Animal Care Committee of the Brigham and Women’s Hospital and Children’s Hospital reviewed and approved the protocols used in the study.

Preparation of RNA and cDNA

Lungs were harvested from fetal mice at embryonic (E) days 12–18 (E12–E18) and from neonatal mice on the day of birth [postnatal (P) day 1 (P1)]. Total RNA was prepared from snap-frozen lung tissue using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNA integrity was evaluated by examination of 18S and 28S bands after separation on ethidium bromide-stained 1.5% agarose-formaldehyde gels. Northern blot analyses were carried out according to standard protocols. cDNA was prepared using SuperScript 2 RNase reverse transcriptase (GIBCO-BRL) according to the manufacturer’s instructions.

RNA Analysis

RT-PCR. Synthetic oligodeoxynucleotides, designed to span at least one intron, were purchased from Oligos Etc (Wilsonville, OR). Primer pairs and PCR conditions are given in Table 2. PCR were carried out using Taq polymerase (Boehringer Mannheim, Indianapolis, IN) as specified by the manufacturer. Briefly, 1 μl of the reverse-transcribed RNA mixture was added to a 50-μl PCR, each of which was subjected to the number of cycles specified in Table 2. Each cycle included denaturation for 1 min at 94°C and annealing for 1.5 min and extension for 2 min at 72°C with a thermal cycler (MJ Research, Watertown, MA). Negative controls consisted of an equal volume of water substituted for the volume of RNA in the RT reaction.

PCR products were analyzed on 1.5% agarose gels containing ethidium bromide and blotted onto nitrocellulose according to standard protocols (5). Internal oligonucleotide probes were end labeled using T4 polynucleotide kinase, hybridized, washed, and exposed to Kodak Biomax film, as described elsewhere (36). These RT-PCR assay conditions were strictly defined to yield semiquantitative results, as previously described (36), resulting in linear detection of positive control bands over a 2-log range of input positive control RNA. Relative amounts of specific mRNAs could subsequently be normalized with actin mRNA in the same RT reaction mixture. Densitometry was carried out by three-dimensional integration of the area of whole bands on autoradiograms with a Scanalyser (Molecular Dynamics, Sunnyvale, CA).

In situ hybridization. Nonisotopic in situ hybridization was carried out to localize mRNAs in tissue sections, as described in detail previously (9). Briefly, fetal mouse lungs were fixed for 3–4 h in 4%}

Table 1. Major groups of BLPs in lung

<table>
<thead>
<tr>
<th>BLP Family</th>
<th>Member 1</th>
<th>Member 2</th>
<th>Member 3</th>
</tr>
</thead>
</table>

BLP, bombesin-like peptide; GRP, gastrin-releasing peptide; RN, ranatensin; NMB, neumedin B; PL, phyllolitorin.

Table 2. PCR primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Temp. °C</th>
<th>No. of Cycles</th>
<th>Band Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP</td>
<td>5′-AAG-CAG-CAG-CTG-AGA-GAG-TA-3′</td>
<td>55</td>
<td>35</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>2′-GAG-AGT-CTA-CCA-ACT-CTG-CC-3′</td>
<td>55</td>
<td>40</td>
<td>334</td>
</tr>
<tr>
<td>GRPR</td>
<td>1′-GTC-AGG-CCA-CGG-CGC-ATC-3′</td>
<td>55</td>
<td>60</td>
<td>403</td>
</tr>
<tr>
<td></td>
<td>2′-ACA-CAG-GGC-GAC-ATC-3′</td>
<td>55</td>
<td>38</td>
<td>335</td>
</tr>
<tr>
<td>NMBR</td>
<td>1′-CAT-GGC-GAA-TGT-CCC-TAA-CAT-C-3′</td>
<td>55</td>
<td>55</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>2′-AGG-GTT-GTT-AAG-GG-CAT-GT-3′</td>
<td>55</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>BRS-3</td>
<td>1′-TGG-GTT-GTT-AAG-GG-GAT-CT-3′</td>
<td>55</td>
<td>55</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>2′-AAC-CAG-ATT-TGC-TGC-TGC-3′</td>
<td>55</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>β-Actin</td>
<td>1′-AAT-GGG-TCA-GAA-GGA-CTG-CAT-3′</td>
<td>55</td>
<td>55</td>
<td>250</td>
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<td></td>
<td>2′-ACG-GTT-GGC-CTT-AGG-GTT-CAG-3′</td>
<td>55</td>
<td>25</td>
<td>250</td>
</tr>
</tbody>
</table>

GRPR, GRP receptor; NMBR, NMB receptor; BRS-3, bombesin receptor subtype 3.
Fig. 1. Ontogeny of bombesin-like peptide (BLP) and BLP receptor mRNAs in developing murine lung. A: lungs were harvested from fetal mice at embryonic days 12–18 (E12–E18) and postnatal days 1, 2, 4, 7, and 14 (P1, P2, P4, P7, and P14). Semiquantitative RT-PCR was carried out as described in MATERIALS AND METHODS. Each lane represents RNA from developing lungs pooled from 2–5 litters (~8–12 pups per litter). β-Actin serves as an internal control to verify RNA integrity and for normalizing experimental data in the same RT reactions. B–D: computerized densitometric analysis was carried out by 3-dimensional integration of RT-PCR bands in A. Relative band intensities were normalized for β-actin in the same RT reaction to correct for differences in mRNA loading and for variation in efficiency of the RT reaction. Results are presented for gastrin-releasing peptide (GRP, solid bars) and GRP receptor (GRPR, open bars) in B, neuromedin B receptor (NMBR) in C, and bombesin receptor subtype 3 (BRS-3) in D.
Determination of \(^3\)H-Labeled DNA and \(^3\)H-Labeled Saturated Phosphatidylcholine

After 44 h of culture with BLPs or dexamethasone, lung organ cultures were incubated with \([\text{\(^3\)H}]\)thymidine (4 \(\mu\)Ci/ml) or \([\text{\(^3\)H}]\)choline (16 \(\mu\)Ci/ml; NEN-Dupont, Boston, MA) for 4 h at 37°C in 5% CO\(_2\)-95% air on a rocking platform, as described previously (29). Tissue viability was checked by histological analyses after 48 h, satisfactory baseline incorporation of \([\text{\(^3\)H}]\)choline and \([\text{\(^3\)H}]\)thymidine, and satisfactory positive control responses with dexamethasone.

Statistical Analyses

Numerical data were analyzed using the unpaired Student’s t-test. Values are means ± SE.

RESULTS

BLP Receptor mRNAs in Developing Murine Lung

To study BLP and BLP receptor gene expression during murine lung ontogeny, tissue was harvested daily from E12–E18 and several postnatal days up to 2 wk of age (P1–P14). The results of semiquantitative RT-PCR analyses of lung RNA are shown in Fig. 1A. Results of relative densitometric analysis, normalized for β-actin, are given in Fig. 1. The GRP gene is expressed throughout embryonic, fetal, and postnatal lung development, with highest levels at E15, E16, and P1 (Fig. 1, A and B). The three BLP receptor mRNAs are expressed in different temporal patterns during ontogeny. GRPR mRNA is detected at E12, peaks at E16, and then falls off to very low levels after birth. NMBR mRNA peaks at E14 but is also present at low to moderate levels from E13 to P1 and at P7. BRS-3 is present at moderate levels at all time points between E12 and P14, with peaks at E15, E16, and P4–P7. Similar results were obtained in three separate experiments using RNA from different pools of fetal mouse lung at all time points (data not shown).

In Situ Hybridization for BLPR mRNAs

In situ hybridization was carried out using antisense cRNA probes to determine localization of GRPR, NMBR, and BRS-3 mRNAs in murine fetal lung at E14, E16, and E18 (Figs. 2 and 3). First, GRPR was localized to airway epithelium and mesenchymal cells at all three time points (Fig. 2). At E14–E16, GRPR was expressed moderately to strongly in the mesenchyme, predominantly around developing airways and blood vessels; a strong signal was also present in the epithelium at E14 and E16. Consistent with the results of RT-PCR analysis, the overall signal for GRPR declined by E18 in the epithelium and mesenchyme.

In contrast, NMBR mRNA was localized predominantly to the diffuse mesenchymal compartment of developing lung at E14 and E18; a moderate NMBR mRNA signal was also present in the airway epithelium at E14 and E16.
Similarly, a strong signal for BRS-3 mRNA was localized to airway epithelium and scattered mesenchymal cells at E14 and E18; at E16, there was a stronger signal for BRS-3 in the airway epithelium. No specific hybridization signal was observed at any time point with any of the sense control probes (Figs. 2 and 3).

Regulation of GRPR, NMBR, and BRS-3 Gene Expression by Dexamethasone and Bombesin

To determine whether agents known to modulate fetal lung development in vivo and in vitro can regulate GRPR, NMBR, or BRS-3 gene expression, semiquantitative RT-PCR analyses were carried out. Again, PCR conditions were determined such that a positive control mRNA was detected within the linear range of the assay and at less than half-maximal signal-to-noise ratio, as previously described (36). We chose dexamethasone as an agent that induces type II cell differentiation and decreased cell proliferation and bombesin as a prototypical BLP that is known to augment cell differentiation and growth. Results of a typical 2-day culture experiment are given in Fig. 4. We carried out densitometric analysis of the RT-PCR bands obtained in four similar experiments. The pooled results of relative densitometric analysis are given in Fig. 4, B–D. Dexamethasone treatment led to a dose-dependent increase in GRPR mRNA levels (Fig. 4B), with a twofold increase in relative amounts of GRPR mRNA with 10 nM dexamethasone ($P < 0.01$). Second, we observed a greater than threefold induction of GRPR gene expression by its specific ligand bombesin at 10 nM ($P < 0.0001$), again in a dose-dependent fashion (Fig. 4C). Finally, NMBR gene expression was suppressed by bombesin in a dose-dependent manner (Fig. 4D), with about half as much NMBR mRNA in cultures treated with 10 nM bombesin as in the negative control ($P < 0.015$). There was no change in BRS-3 mRNA levels with any tested dose of dexamethasone or bombesin (data not shown).

To determine whether all three groups of BLPs can promote type II cell differentiation and/or generalized cell proliferation in fetal mice, lung explants were cultured with BLPs from each of the three recognized BLP families (Table 1) vs. dexamethasone as a control. Peak $[^3H]$thymidine incorporation occurred on E18 (see Fig. 6). The maximal effect was observed with 0.1 nM GRP (−75% increase over baseline). GRP also elicited significant responses of smaller magnitude on E17 (<15% increase at 0.1–1.0 nM GRP; Fig. 5). In parallel cultures from the same litters of mice, NMB stimulated $[^3H]$thymidine incorporation ~20% over baseline at 0.1–1.0 nM on E18 (Fig. 5), with a peak effect (~70% increase over baseline) observed at 10 nM. NMB also elicited significant responses of smaller magnitude on E17 (<25% increase at 0.1–1.0 nM NMB; Fig. 5). L8PL had minimally significant effects on cell proliferation (<15% inhibition with 1.0–10 nM L8PL on E17 and <10% stimulation with 0.1–1.0 nM L8PL on E18). Low-dose (1.0–10 nM) dexamethasone stimulated thymidine incorporation (~10–20%) on E17 and E18. At higher doses, dexamethasone inhibited $[^3H]$thymidine incorporation on E17 (>50% inhibition with 100 nM) and E18 (>25% inhibition with 10 nM).

In contrast, maximal effects on stimulating choline incorporation were observed on E17 (Fig. 5) and E18 (Fig. 6). GRP at 0.1 nM induced an ~20% increase in choline incorporation on E17 and E18. The magnitude of these GRP effects was similar to that of the 0.1 nM dexamethasone control on E17 (Fig. 5), whereas the response to 10 nM dexamethasone on E18 was of greater magnitude (>30%). NMB inhibited choline incorporation ~15–25% on E17 at 1.0, 10, and 100 nM; L8PL was inhibitory (~35%) only at 100 nM. In contrast, on E18, 10 nM NMB stimulated choline incorporation to the same extent (~30%) as the 10 nM dexamethasone control. High-dose (100

Fig. 3. In situ hybridization analysis for BRS-3 in E18 mouse lung. In situ hybridization for BRS-3 was carried out with 3-μm sections of E14, E16, and E18 mouse lung using a homologous BRS-3 antisense cRNA probe. Negative control for in situ hybridization was carried out with closely adjacent serial sections of E18 mouse lung using the corresponding BRS-3 sense cRNA probe. No hybridization signal was observed with BRS-3 sense cRNA. Arrows, conducting airways; v, medium-sized blood vessels; *, pleural surface.
nM) L8PL inhibited choline incorporation on E17, whereas low- and high-dose (1.0 and 100 nM) L8PL stimulated choline incorporation on E18.

**DISCUSSION**

The present study demonstrates that the three cloned BLP receptor genes are expressed in developing murine lung. However, there are striking differences between these genes with regard to their ontogeny of gene expression, relative localization of mRNAs, and regulation by dexamethasone and bombesin. In a similar fashion, the three groups of bombesin-related peptides have different functional effects in modulating cell proliferation and type II cell differentiation in fetal lung explants.

The temporal and spatial distribution of gene expression has been reported to differ during development between different members of other gene families (17, 25). The ontogeny of GRP and GRPR genes in fetal mouse lung has been previously analyzed in our laboratory (19, 29). To complete the assessment of BLP receptor gene expression in developing mouse lung, we demonstrate mRNAs encoding NMBR and BRS-3 as well. GRP, GRPR, and BRS-3 mRNAs are detectable as early as E12 and E13; NMBR mRNA is detectable at E13 and reaches peak levels on E14. This timing coincides with the period of branching morphogenesis, suggesting a potential regulatory role for GRP and the three BLP receptors in early organogenesis, as we previously addressed (19). Whereas GRPR mRNA levels in the lung drop off markedly shortly after birth, NMBR and BRS-3 mRNAs are sustained.

Others have shown that glucocorticoids can inhibit GRPR mRNA levels in pancreatic acinar cells (18). Not infrequently, mesenchymal cells and epithelial cells can have opposite responses to the same ligand (2, 14). The well-recognized effect of dexamethasone on promoting fetal lung maturation could be
mediated in part by dexamethasone-mediated upregulation of GRPR on fibroblasts and enhanced signaling through the GRPR pathway (9). The opposite regulation of GRPR and NMBR gene expression is interesting, because GRP promotes but NMB inhibits choline incorporation in developing E17 lung. Teleologically, this dichotomy may reflect ligand-specific (bombesin) upregulation of the preferred native receptor (GRPR) and decreased levels of secondary receptors with lower affinity (NMBR).

It should be emphasized that the identity of the native BRS-3 ligand is unknown. Furthermore, bombesin, NMB, and PL have very low affinity for BRS-3; hence, the effects of these three peptides on fetal lung are unlikely to be mediated via BRS-3. This is further supported by our observation that bombesin does not alter BRS-3 gene expression in fetal lung explants. In addition, neither a mammalian PL nor a mammalian PL receptor has been identified. The effects of amphibian PL in the present study could be mediated in part by GRPR, but it is likely that additional novel BLP receptors are involved as well.

Our physiological data suggest the possibility that GRPR and NMBR play different roles in lung development. Low- and high-dose GRP induce a marked increase in cell proliferation on E18 that is likely to be mediated via GRPR and NMBR and, potentially, other novel BLP receptors. In contrast, only low-dose GRP stimulates significant choline incorporation on E17, supporting a unique role for GRPR in type II cell differentiation. This effect on type II cell differentiation is likely to be mediated via GRPR on fetal lung mesenchymal cells, because we previously demonstrated a requirement for these cells to elicit the GRP effect on type II cells in culture (8). In contrast, low-dose NMB promotes only thymidine incorporation, consistent with a major role for NMBR in cell proliferation. Higher-dose (10 nM) NMB stimulates a greater degree of cell proliferation (~70%), similar to the effect of GRP at low and high doses, suggesting that 10 nM NMB may function via GRPR and/or other novel BLP receptors. In contrast to GRP, NMB (1.0–100 nM) only inhibits choline incorporation on E17, whereas 10 nM NMB is stimulatory on E18. The latter effect is likely to be mediated via GRPR.

The effects of BLPs on E17 lung explants (Fig. 5) differ markedly from their effects on E18 cultures (Fig. 6). These differences are likely to reflect large changes in developing murine lung between E17 and E18. On E17, the lung is in the late canalicular phase, during which there is widespread ingrowth of capillaries into developing alveolar septa as well as the induction of type II cell differentiation. On E18, the lung is entering the saccular phase: type II cell differentiation is more advanced, and structural remodeling predominates, together with intense epithelial cell proliferation. These developmental differences may explain why low-dose GRP promotes cell proliferation most intensely on E18, when GRPR is expressed predominantly in the proliferating epithelium. In contrast, low-dose GRP stimulates type II cell differentiation most strongly on E17, when GRPR mRNA is most strongly expressed in the mesenchyme.

It is unlikely that a PL receptor plays a major role in the observed responses. L8PL has only weak (<15%) effects on thymidine incorporation at any dose. L8PL had more than a marginal effect on choline incorporation at 100 nM, which could be mediated via NMBR (33). Previously, we demon-
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