Prostacyclin analogs stimulate VEGF production from human lung fibroblasts in culture

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Kamio K, Sato T, Liu X, Sugiuira H, Togo S, Kobayashi T, Kawasaki S, Wang X, Mao L, Ahn Y, Holz O, Magnussen H, Rennard SI. Prostacyclin analogs stimulate VEGF production from human lung fibroblasts in culture. Am J Physiol Lung Cell Mol Physiol 294: L1226–L1232, 2008. First published April 18, 2008; doi:10.1152/ajplung.00129.2007.—Prostacyclin is a short-lived metabolite of arachidonic acid that is produced by several cells in the lung and prominently by endothelial cells. It increases intracellular cAMP levels activating downstream signaling thus regulating vascular mesenchymal cell functions. The alveolar wall contains a rich capillary network as well as a population of mesenchymal cells, i.e., fibroblasts. The current study evaluated the hypothesis that prostacyclin may mediate signaling between endothelial and mesenchymal cells in the alveolar wall by assessing the ability of prostacyclin analogs to modulate fibroblast release of VEGF. To accomplish this study, human lung fibroblasts were cultured in routine culture on plastic support and in three-dimensional collagen gels with or without three prostacyclin analogs, carboxyprostacyclin, iloprost, and beraprost, and the production of VEGF was evaluated by ELISA and quantitative real-time PCR. Iloprost and beraprost significantly stimulated VEGF mRNA levels and protein release in a concentration-dependent manner. These effects were blocked by the adenylate cyclase inhibitor SQ-22536 and by the protein kinase A (PKA) inhibitor KT-5720 and were reproduced by a direct PKA activator but not by an activator of exchange protein directly activated by cAMP (Epac), indicating that cAMP-activated PKA signaling mediated the effect. Since VEGF serves to maintain the pulmonary microvasculature, the current study suggests that prostacyclin is part of a bidirectional signaling network between the mesenchymal and vascular cells of the alveolar wall. Prostacyclin analogs, therefore, have the potential to modulate the maintenance of the pulmonary microcirculation by driving the production of VEGF from lung fibroblasts.

THE ALVEOLAR WALL IS AN EXTREMELY thin and attenuated structure. Nevertheless, it has a complex organization containing a number of structural cells, including endothelial cells, type I and II alveolar cells, and fibroblasts/myofibroblasts. Alveolar fibroblasts, which comprise as much as 37% of alveolar cells, are believed to be the main cell source of alveolar connective tissue (11). It is now clear that fibroblasts are also a rich source of growth factors and inflammatory mediators that can regulate the activity of other resident and recruited cells in the lung.

VEGF was first identified as a vascular permeability factor in tumor cell-conditioned medium (34) and independently as a mitogenic activity for vascular endothelial cells (13, 34). The lung has VEGF expression that is among the highest for parenchymal organs (8, 14). It is likely that VEGF production in the lung serves to maintain the integrity of the pulmonary microvasculature, which is, in turn, required for maintenance of the integrity of the alveolar wall. Loss of VEGF signaling, in this context, has been associated with the development of pulmonary emphysema (19). However, the source of VEGF within the alveolar wall and the regulation of its production is not fully understood.

Prostacyclin, which is produced in the lung by endothelial cells, fibroblasts, and type II alveolar cells, has been reported to induce VEGF production from rat intestinal epithelial cells (36) and from the human monocytoid-like cell line THP-1 (25). The current study was designed to determine if prostacyclin could modulate VEGF production by human lung fibroblasts. To investigate this, three prostacyclin analogs, carboxyprostacyclin, iloprost, and beraprost, were assessed for their effects on VEGF production by human lung fibroblasts. The effect of these analogs, moreover, was evaluated both in routine culture on plastic supports and using culture in three-dimensional collagen gels (3-D gels).

MATERIALS AND METHODS

Materials. Commercially available reagents were obtained as follows: DMEM and FCS were from Invitrogen (Grand Island, NY); carboxyprostacyclin, iloprost, and beraprost were from Cayman Chemical (Ann Arbor, MI); anti-human VEGF antibody and biotinylated anti-human VEGF antibody were from R&D Systems (Minneapolis, MN); horseradish peroxidase (HRP)-streptavidin conjugate was from Zymed Laboratories (South San Francisco, CA); 8-pCPT-2’-O-Me-CAMP, dibutyryl cAMP, and 3,3,5,5-tetramethylbenzidine (TMB) were from Sigma (St. Louis, MO); KT-5720, 6-Bnz-cAMP, and SQ-22536 were from Calbiochem (San Diego, CA).

Cell culture. Human fetal lung fibroblasts (HFL-1, lung, diploid, human) were obtained from the American Type Culture Collection (ATCC).
(CCL-153; Rockville, MD). In addition, fibroblasts were cultured from the lungs of patients undergoing thoracic surgery for removal of tumors. In all cases, subjects had normal lung function. Fibroblast cultures were initiated from normal-appearing areas of the pulmonary parenchyma in a region as far as possible from the tumor that was free of pleura or large airways. After isolation, cells were aliquoted, frozen, and shipped to the University of Nebraska Medical Center where all in vitro experiments were performed. The isolation of these cells has previously been described (15). The cells were cultured on 100-mm tissue culture dishes (BD Falcon; Becton Dickinson Laboratoryware, Lincoln Park, NJ) with DMEM supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin sulfate (penicillin-streptomycin, Invitrogen), and 2.5 μg/ml amphotericin B (Geneva Pharmaceuticals, Dayton, NJ). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and passed every 3–5 days at a 1:4 ratio. In all experiments, cells between passages 14 and 18 were used. To evaluate VEGF production in monolayer culture, cells were seeded in six-well tissue culture plates at a cell density of 1 × 10⁵ per milliliter. At 90% confluence, cells were serum-starved for 6 h and then treated with various concentrations of prostacyclin analogs in serum-free DMEM (SF-DMEM). The supernatants of monolayer culture were harvested 48 h later and stored at −80°C until assayed. When used, KT-5720 (1 × 10⁻⁷ M; Refs. 5, 10) or the adenylate cyclase inhibitor SQ-22536 (1 × 10⁻⁴ M) were added 1 h before the addition of prostacyclin analogs.

Three-dimensional collagen gel culture. Native type I collagen [rat tail tendon collagen (RTTC)] was extracted from rat tail tendons by a previously published method (12, 27). Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were removed carefully. After repeated washing with Tris-buffered saline (0.9% NaCl and 10 mM Tris, pH 7.5), dehydration and sterilization with 50%, 75%, and 95%, and pure ethanol were performed. Type I collagen was then extracted in 6 mM hydrochloric acid at 4°C. Protein concentration was determined by weighing a lyophilized aliquot from each lot of collagen solution. SDS-PAGE demonstrated no detectable proteins other than type I collagen.

Confluent fibroblasts were detached by trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA–Na; GIBCO, Grand Island, NY) and resuspended in SF-DMEM containing soybean trypsin inhibitor (Sigma). The cell number was then counted with a Coulter Counter. Collagen gels were prepared by mixing the appropriate amount of RTTC, distilled water, 4× concentrated DMEM, and cell suspension so that the final mixture resulted in 0.75 mg/ml collagen, 3 × 10⁵ fibroblasts per milliliter of gel, and a physiological ionic strength of 1× DMEM, pH 7.4 (27). Fibroblasts were routinely added last to minimize damage during the preparation of collagen gels. A 500-μl portion of the gel solution was then cast into each well of a 24-well tissue culture plate with a 2-cm² growth area (BD Falcon). Gelation occurred in 20 min at room temperature, after which the gels were released from the surface of the culture well using a sterile spatula and transferred into 60-mm tissue culture dishes (3 gels in each dish) containing 5 ml of SF-DMEM with various concentrations of prostacyclin analogs and incubated at 37°C, 5% CO2. Media were harvested on day 2 and stored at −80°C until assayed. When used, KT-5720 (1 × 10⁻⁷ M) or SQ-22536 (1 × 10⁻⁴ M) were added 1 h before the addition of prostacyclin analogs.

Measurement of VEGF by ELISA. VEGF in the media of monolayer and 3-D gel cultures was quantified by ELISA. Ninety-six-well plates were coated with 50 ng/ml monoclonal anti-human VEGF antibody at 4°C overnight. After washing three times, standards and samples were added and incubated at room temperature for 2 h. After washing three times, bound antigen was detected with 100 ng/ml biotinylated anti-human VEGF antibody for 1 h at room temperature followed by washing and then HRP-streptavidin conjugate (1:20,000 dilution) for 1 h at room temperature. After a final washing, bound HRP was detected with TMB (37). The reaction was stopped with 1 M H₂SO₄, and the product was quantified at 450 nm with a microplate reader (Bio-Rad, Hercules, CA).

RNA preparation and complementary DNA synthesis. To determine whether changes in VEGF mRNA levels were present, quantitative real-time PCR was performed. Cells were cultured until confluence and serum-starved for 2 h. Then, cells were exposed to 1 × 10⁻⁶ M carboprostacyclin, iloprost, or beraprost for 0.5, 1, 2, 4, and 6 h. Total RNA was isolated by the single-step guanidinium-thiocyanate-phenol-chloroform extraction procedure of Chomczynski and Sacchi (9), and the total amount was quantified spectrophotometrically (Shimadzu Scientific Instruments, Columbia, MD). One microgram of total RNA was treated with RNase-free DNase I following the manufacturer’s instructions (Invitrogen) for 15 min at room temperature to remove possible contaminating genomic DNA. The reaction was then stopped with 25 mM EDTA by heating at 65°C for 10 min followed by 95°C for 5 min. For cDNA synthesis, ~400 ng of DNase-treated RNA was transcribed with cDNA transcription reagents (Applied Biosystems, Foster City, CA) by using random hexamers, and the cDNA was used for quantitative real-time PCR.

Quantitative real-time PCR. Gene expression was measured with the use of an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) as described previously (6). TaqMan Gene Expression Assays, which includes predesigned primers and probes for the detection of VEGF mRNA (Hs00173626_m1), were purchased from Applied Biosystems. Probes were labeled at the 5’ end with the reporter dye molecule 6-carboxy-fluorescein (FAM) and at the 3’ end with the quencher dye molecule 6-carboxytetramethyl-rhodamine (TAMRA). RNA was simultaneously tested using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). Quantitative real-time PCRs of cDNA specimen were conducted in a total volume of 50 μl with 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and primers at 900 nm and probes at 250 nm. Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Data were normalized to the amount of rRNA from the same preparations and expressed as fold of control.

Statistical analysis. Data were expressed as means ± SE. Experiments with multiple comparisons were evaluated by one-way ANOVA followed by Tukey post hoc test to adjust for multiple comparisons. Probability values of <0.05 were considered significant.

RESULTS

Effect of prostacyclin analogs on VEGF release by human lung fibroblasts. To determine whether prostacyclin analogs could alter VEGF production, VEGF release into monolayer culture media was evaluated by ELISA. After 2 days, culture with various concentrations of prostacyclin analogs (1 × 10⁻⁹ to 1 × 10⁻⁶ M) iloprost and beraprost increased VEGF production significantly in a concentration-dependent manner (P < 0.05; Fig. 1). In contrast, carbaprostacyclin had a modest stimulatory effect that was not clearly concentration dependent. To confirm that the effect of prostacyclin analogs was a general property of lung fibroblasts, cells isolated from the lungs of three different adult subjects were also evaluated. Although there were some quantitative differences among the strains, all three of the strains responded to all of the prostacyclin analogs (all 1 × 10⁻⁶ M) with an augmentation of VEGF release, and the magnitude of the response was similar for all strains of cells tested (Table 1).

Role of adenylate cyclase in the stimulation of VEGF release by iloprost and beraprost. To assess the role of adenylate cyclase in the stimulation of VEGF release induced by iloprost and beraprost, we investigated whether the adenylate cyclase inhibitor SQ-22536 inhibits VEGF release from lung fibro-
Prostacyclin analogs modulate fibroblast-mediated VEGF production: an experimental study

Fig. 1. Effect of prostacyclin analogs on VEGF release into monolayer culture media. Fibroblasts were cultured until 90% confluence in 6-well tissue culture plates, after which media were changed to serum-free DMEM (SF-DMEM) with varying concentrations of prostacyclin analogs. After 2 days, monolayer plates, after which media were changed to serum-free DMEM (SF-DMEM) media. Fibroblasts were cultured until 90% confluence in 6-well tissue culture plates, after which media were harvested and assayed for VEGF by ELISA. Vertical axis: concentration of prostacyclin analog (molar). The data presented are means ± SE from 3 separate experiments, each performed in duplicate. *P < 0.05 compared with the values of control.

Table 1. Effect of prostacyclin analogs on VEGF release by various strains of human lung fibroblasts

<table>
<thead>
<tr>
<th>Cell Strain</th>
<th>Control</th>
<th>Carboprostacyclin</th>
<th>Iloprost</th>
<th>Beraprost</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFL-1</td>
<td>33.3 ± 4.04</td>
<td>47.7 ± 5.02</td>
<td>73.2 ± 1.68*</td>
<td>58.9 ± 1.40*</td>
</tr>
<tr>
<td>05-383</td>
<td>39.5 ± 3.67</td>
<td>85.1 ± 3.17*</td>
<td>98.6 ± 3.06*</td>
<td>74.0 ± 1.07*</td>
</tr>
<tr>
<td>06-108</td>
<td>23.6 ± 2.23</td>
<td>93.6 ± 1.86*</td>
<td>111.4 ± 10.7*</td>
<td>87.6 ± 3.43*</td>
</tr>
<tr>
<td>06-109</td>
<td>41.9 ± 7.89</td>
<td>207.1 ± 6.00*</td>
<td>212.6 ± 12.0*</td>
<td>191.7 ± 7.31*</td>
</tr>
</tbody>
</table>

Values are means ± SE (picograms produced per 10⁵ cells per day) from 3 separate experiments, each performed in duplicate. *P < 0.05 compared with the value of control. 05-383, 06-383, and 06-109 are the strains of normal lung fibroblasts. HFL-1, human fetal lung fibroblasts.

cAMP signaling pathways and VEGF release. cAMP can activate several downstream signaling pathways including the classical protein kinase A (PKA) pathway and the more recently described exchange protein directly activated by cAMP (Epac) pathway. To evaluate the role of these pathways in VEGF release from lung fibroblasts, we used the PKA-specific agonist 6-Bnz-cAMP and the Epac-selective agonist 8-pCPT-2′-O-Me-cAMP. As a control, we used the nonselective cAMP analog dibutyryl cAMP. 6-Bnz-cAMP and dibutyryl cAMP both significantly induced VEGF release from lung fibroblasts, whereas, in contrast, 8-pCPT-2′-O-Me-cAMP did not (Fig. 3).

To further confirm the role of PKA, we used an inhibitor of PKA, KT-5720. This antagonist blocked the stimulatory activity of the nonselective cAMP analog dibutyryl cAMP. In addition, preincubation of fibroblasts in monolayer culture with KT-5720 (1 × 10⁻⁷ M) for 1 h before the addition of prostacyclin analogs (1 × 10⁻⁶ M) significantly inhibited the stimulation of VEGF release by iloprost and beraprost (Fig. 4). There was no effect of the PKA inhibitor on the release of VEGF in either control, unstimulated cells or in cells treated with carbaprostacyclin, which themselves did not differ from control.

Effect of prostacyclin analogs on VEGF mRNA expression. To determine whether the stimulation of VEGF release was associated with a change in gene expression, quantitative

Fig. 2. Adenylate cyclase mediates prostacyclin analog stimulation of VEGF production. Fibroblasts cultured in monolayer were incubated without (open bars) or with (closed bars) the adenylate cyclase inhibitor SQ-22536 (1 × 10⁻⁴ M) for 1 h before treatment with prostacyclin analogs (1 × 10⁻⁶ M). After 2 days, culture media were harvested and assayed for VEGF by ELISA. Vertical axis: VEGF production (picograms produced per 10⁵ cells per day). Horizontal axis: conditions. The data presented are means ± SE from 3 separate experiments, each performed in duplicate. *P < 0.01 compared with nontreated groups.

Fig. 3. cAMP signaling pathway and VEGF stimulation. Fibroblasts were incubated with or without 8-pCPT-2′-O-Me-cAMP, 6-Bnz-cAMP, or dibutyryl cAMP (all 5 × 10⁻⁴ M). In addition, cells were also treated with KT-5720 (1 × 10⁻⁷ M) for 1 h before treatment with dibutyryl cAMP. After 2 days, culture media were harvested and assayed for VEGF by ELISA. Vertical axis: VEGF production (picograms produced per 10⁵ cells per day). Horizontal axis: conditions. The data presented are means ± SE from 3 separate experiments, each performed in duplicate. †P < 0.001 compared with nontreated and 8-pCPT-2′-O-Me-cAMP-treated groups; #P < 0.001 compared with KT-5720-untreated groups.
DISCUSSION

The current study demonstrates that prostacyclin analogs, iloprost and beraprost, stimulate VEGF production by human lung fibroblasts. This was observed in both the widely used primary strain of HFL-1 and in three separate freshly isolated primary adult human lung fibroblasts. Stimulation was observed in both monolayer and 3-D collagen gel culture. The stimulatory effect appears to be mediated through cAMP activation of the PKA pathway as an inhibitor of adenylyl cyclase and an inhibitor of PKA blocked the stimulation of VEGF release without affecting VEGF release by control cells. In parallel, a nonselective cAMP agonist and a PKA-selective agonist stimulated VEGF release whereas an Epac-selective agonist did not. Finally, the stimulatory effect of iloprost and beraprost is associated with an increase in VEGF mRNA expression.

Many lung diseases are associated with alterations in pulmonary microvasculature. This suggests that alterations in real-time PCR assay was performed. Consistent with the results of protein expression assessed by ELISA, iloprost and beraprost at the concentration of $1 \times 10^{-6}$ M stimulated VEGF mRNA expression in HFL-1 fibroblasts (Fig. 5). The peak increase in VEGF mRNA was observed at 1 h and was $2.68 \pm 0.22$- and $3.74 \pm 0.28$-fold of control following stimulation by iloprost ($P < 0.01$; Fig. 5B) and beraprost ($P < 0.01$; Fig. 5C). In contrast, carbaprostacyclin did not appear to affect the VEGF mRNA expression (Fig. 5).

Effect of prostacyclin analogs on VEGF release from fibroblasts cultured in 3-D gels. Prostacyclin analogs have been shown to inhibit the contraction of 3-D collagen gels in a concentration-dependent manner (17). However, the response of fibroblasts to exogenous mediators in 3-D culture can differ from the response in monolayer culture. To investigate whether prostacyclin analogs stimulate VEGF production in 3-D gel culture media, fibroblasts were cultured in 3-D gels with various concentrations of prostacyclin analogs (1-10^{-6} M). Media were harvested on day 2 and analyzed by ELISA for VEGF. The effect of prostacyclin analogs on VEGF release in 3-D gel culture resembled the effect in monolayer culture. Iloprost and beraprost increased VEGF production in a concentration-dependent manner with a peak at the highest concentration tested ($1 \times 10^{-6}$ M; $P < 0.05$; Fig. 6). The effect of carbaprostacyclin on VEGF release was modest and not statistically significant.

We further investigated whether KT-5720 inhibits VEGF production stimulated by iloprost and beraprost in 3-D gel culture media. Preincubation of HFL-1 fibroblasts in 3-D gel culture with KT-5720 ($1 \times 10^{-5}$ M) for 1 h before the addition of prostacyclin analogs ($1 \times 10^{-6}$ M) inhibited the production of VEGF stimulated by iloprost and beraprost (Fig. 7).
VEGF within the lung may play a pathogenic role. Consistent with this, Koyama et al. (24) have demonstrated a decrease in VEGF levels in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis and with pulmonary fibrosis associated with connective tissue disease and sarcoidosis when compared with nonsmoking volunteers. Similarly, Kasahara et al. (18) has demonstrated a reduction in both VEGF and VEGF receptors in the lungs of patients with emphysema. In animal models, blockade of VEGF signaling, either pharmacologically or immunologically, has been associated with endothelial cell apoptosis and the development of emphysema (19). This supports the concept proposed by Liebow (26) that maintenance of endothelial viability is required to maintain alveolar structure. The current study suggests that prostacyclin, by stimulating fibroblast production of VEGF, could play a role in the maintenance and alteration of the pulmonary microcirculation.

Within the lung, a number of cells can produce prostacyclin. Among these, alveolar type II epithelial cells (31), endothelial cells (29, 33), and fibroblasts (32) are particularly rich sources. The current study did not evaluate the production of prostacyclin by lung fibroblasts but rather demonstrated that these cells can respond to prostacyclin by producing VEGF. The production of prostacyclin within the lung is believed to modulate both the contractile tone and metabolic activity of pulmonary arterial smooth muscle. The alveolar wall, in contrast, lacks smooth muscle but contains a population of fibroblasts/myofibroblasts in close proximity to endothelial cells and alveolar epithelial cells (7). The ability of prostacyclin to stimulate fibroblast production of VEGF, therefore, could play a key role in coordinating the cellular responses required for the maintenance of normal alveolar structures.

Stimulation of VEGF production by prostacyclin analogs has been demonstrated by other groups using cell lines derived from different types of cells. Lederle et al. (25) showed that iloprost induced VEGF in a human monocytic cell line. Similarly, Buchanan et al. (4) reported that prostacyclin induced VEGF expression in a rat intestinal epithelial cell line. The cAMP-PKA signaling pathway mediated the effect in both cell types, and an increase in VEGF mRNA levels was observed. Consistent with this, the cAMP-PKA pathway was demonstrated to mediate an increase in VEGF in response to β-adrenoreceptor in mouse brown adipocytes (16). The current study extends these observations to demonstrate that normal human lung fibroblasts also respond to prostacyclin with an increase in VEGF. Moreover, lung fibroblasts responded in both monolayer and 3-D gel culture. We confirmed that PKA mediates stimulation of VEGF production and established that the alternative cAMP signaling pathway Epac does not play a role. As a number of responses of cultured cells can vary with culture condition, the current study provides evidence supporting a role for the alveolar mesenchyme to direct the maintenance of the alveolar microcirculation.

Because of their relaxant action on pulmonary arterial smooth muscle, a number of prostacyclin analogs have been developed. Among these, iloprost and beraprost have been used for the treatment of patients with pulmonary arterial hypertension (1). Both of these are relatively specific for the prostaglandin I2 (prostacyclin, IP) receptor. In contrast, carboprostacyclin, which is also active on the IP receptor, is relatively less specific, having action on the prostanoit EP1 and EP3 receptors as well (2, 20). We have demonstrated expression of all three receptors in cultured HFL-1 cells by immunoblotting (data not shown). Classically, the IP receptor signals through Gs by activating adenyl cyclase leading to cAMP-mediated signaling. The actions of iloprost and beraprost observed in the current study are consistent with this signaling mechanism. The lack of activity observed with carboprostacyclin may be due to signaling through other receptors and pathways.
The ability of prostacyclin to modulate fibroblasts through a cAMP-mediated pathway suggests that responses in addition to VEGF production may also be regulated. In this regard, a number of agents that increase cAMP have been demonstrated to inhibit several profibrotic responses of fibroblasts, including chemotaxis (21–23), proliferation (3, 30, 38), production of ECM (30, 38), and contraction of 3-D collagen gels (17, 23, 28). Prostacyclin analogs, acting through a PKA-dependent pathway, have recently been demonstrated to inhibit fibroblast-mediated contraction of 3-D collagen gels (17). Consistent with a role for prostacyclin and the IP receptor in modulating tissue repair and remodeling is the report that iloprost can modulate the development of lung fibrosis (35). This suggests that prostacyclin may function as an overall regulator of fibroblast maintenance of tissue structure.

In summary, the current study demonstrates that prostacyclin analogs can modulate the production of VEGF by normal human lung fibroblasts. This pathway has the possibility of regulating the maintenance and alteration of pulmonary microvasculature. As such, this provides a mechanism that could play a role in diseases of the alveolar wall, including pulmonary fibrosis and emphysema.

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