Regulation of Heparanase Gene Expression by Estrogen in Breast Cancer

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ABSTRACT

Numerous epidemiological studies clearly suggest that estrogen is one of the main driving forces in breast tumorigenesis, but precise mechanisms of cancer promotion by estrogen remain poorly understood. Classically, tumorigenic effects of estrogen have been attributed to its ability to directly promote the proliferation of breast cancer cells. In addition to abnormal proliferation, interactions between tumor cells and surrounding stromal components (e.g., enzymatic remodeling and degradation of extracellular matrix) are critical for cancer progression, angiogenesis, and metastasis. We now report that in breast carcinomas, estrogen may promote these pathological tumor-stromal interactions through up-regulation of heparanase gene expression. Heparanase is an endoglycosidase degrading heparan sulfate, of the basement membrane and extracellular matrix. This cleavage affects tumor-stromal interaction, neovascularization, local invasion, and metastatic spread. However, little is known about transcriptional regulation of the heparanase gene. We identified four putative estrogen response elements in the heparanase promoter region and found that transcription of a luciferase reporter gene driven by the heparanase promoter was significantly increased in estrogen-receptor positive MCF-7 human breast carcinoma cells after estrogen treatment. Estrogen-induced heparanase mRNA transcription in estrogen receptor-positive, but not in estrogen receptor-negative, breast cancer cells, confirmed the promoter study data. The estrogen effects on heparanase mRNA expression levels were abolished in the presence of the pure antiestrogen ICI 182,780, indicating that the classic estrogen receptor pathway is involved in transcriptional activation of heparanase. In vivo, exposure to estrogen augmented levels of heparanase protein in MCF-7 cells embedded in Matrigel plugs and correlated with increased plug vascularization. Collectively, our data suggest a new molecular pathway through which estrogen, independent of its proliferative effect, may induce heparanase overexpression and, thus, promote tumor-stromal interactions, critical for breast carcinoma development and progression.

INTRODUCTION

Enhanced activity of estrogen signaling is thought to be one of the major driving forces in breast cancer development (1). The tumor-promoting action of estrogen has been primarily attributed to its ability to directly enhance the proliferation of breast cancer cells (1–5). However, regulation of cell proliferation represents just one aspect of contribution of estrogen to breast tumorigenesis. Of much importance is the identification of “novel” downstream estrogen effectors implicated in tumorigenesis, regardless of their association with proliferation. Moreover, such newly identified estrogen effectors are expected to become important targets for therapy of breast cancer and other estrogen-related pathological conditions (2).

It is well recognized now that, apart of enhanced proliferation, abnormal interactions between tumor cells and surrounding extracellular stromal elements are critical for cancer progression, angiogenesis, and metastasis (6, 7). The molecular basis of such interactions involves enzymatic remodeling and degradation of the extracellular matrix (ECM; reviewed in Ref. 6). Several lines of evidence indicate that estrogen and related compounds could participate in the regulation of this process (8–11). In the present study, we investigated the effects of estrogen on heparanase expression as a possible mechanism of such regulation.

Heparanase (endo-β-D-glucuronidase) degrades heparan sulfate (HS), the main polysaccharide component of the basement membrane and ECM (12–17). HS plays a key role in the self-assembly and integrity of the ECM multi-molecular architecture (18, 19). Malignant tumor growth, neovascularization, and metastasis represent invasive processes that involve enzymatic disintegration of the ECM. It is not surprising, therefore, that cleavage of HS by heparanase is a critical event in cancer development and progression (12–17). In particular, heparanase is tightly involved in tumor angiogenesis (13, 20–22). Apart from its direct contribution to ECM degradation and endothelial cell sprouting, heparanase elicits an indirect neovascular response by releasing HS-bound angiogenic factors stored in the ECM (i.e., basic fibroblast growth factor), making them bioavailable for signaling receptors on the endothelial cell surface (21).

Heparanase is closely associated with breast tumorigenesis. Its expression and enzymatic activity correlate with the aggressiveness of human breast carcinoma cell lines, and there is increased expression of the heparanase gene and protein are preferentially expressed in human breast tumors when compared with the corresponding normal tissue (12, 14). In breast carcinoma patients, heparanase expression is associated with the metastatic potential of the tumor (23). Mammary tumors produced by MCF-7 breast carcinoma cells, genetically engineered to overexpress heparanase cDNA, grew faster, were 4–5-fold larger, and were more vascularized than tumors produced by control MCF-7 cells, further demonstrating the important role of heparanase in breast cancer progression. In the present study, we investigated estrogen regulation of heparanase expression in breast carcinoma cells. We identified estrogen response element homologous sequences in the promoter region of the heparanase gene, and found that estrogen stimulates transcriptional activity of the heparanase promoter. Estrogen treatment induced a significant increase in the levels of heparanase mRNA and protein in vitro and in vivo, respectively. Using an in vivo breast cancer angiogenesis model, we found that the estrogen-mediated increase in heparanase protein levels led to augmented neovascularization.

Collectively, our data suggest that transcriptional activation of heparanase may represent a new molecular mechanism through which estrogen, affecting pathological tumor-stromal interactions (i.e., ECM degradation, angiogenesis), may contribute to the development and progression of breast cancer.
MATERIALS AND METHODS

Cell Culture. MCF-7 and MDA-MB-231 human breast carcinoma cells were obtained from the American Type Culture Collection. T47D cell line was kindly provided by Dr. E. Ginsburg (NIH, Bethesda, MD). Cells were routinely maintained in DMEM (MDA-MB-231, T47D cells) or RPMI 1640 (MCF-7 cells) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), at 37°C and 5% (MCF-7) or 10% (MDA-MB-231 and T47D) CO₂. Before estrogen treatment, cells were maintained for 96 h in phenol red-free medium supplemented with charcoal-stripped fetal bovine serum (HyClone, Logan, UT). Then, medium was changed to serum-free medium and estrogen with or without inhibitors was added. Estrogen (17β-estradiol) and 4-hydroxy-tamoxifen were obtained from Sigma (St. Louis, MO) and dissolved in absolute ethanol. Pure estrogen receptor antagonist ICI 182,780 was purchased from Toecris Cookson (Bristol, United Kingdom) and dissolved in DMSO. Control cultures were treated with the corresponding vehicle (0.1% ethanol or DMSO).

Reporter Construct Transfections and Luciferase (LUC) Assay. The 1.9-kb human heparanase promoter region [HPSE (−1791/+109)]-LUC] was subcloned upstream of the LUC gene in a pGL2 basic reporter plasmid (Promega, Madison, WI). Cells were seeded into 48-well plates and maintained for 4 days in phenol-red-free medium supplemented with 10% charcoal-treated fetal bovine serum (HyClone, Logan, UT). Transfections were performed using the standard protocol for LipofectAMINE PLUS Reagent (Life Technologies, Inc., Grand Island, NY). Briefly, reporter construct (0.16 μg/well) was mixed with LipofectAMINE (0.4 μl/well) and PLUS reagent (1.6 μl/well) and added to cells in a total volume of 0.1 ml serum-free medium. Control cells were transfected with basic pGL2 plasmid containing LUC gene alone (without promoter). After 3 h, cells were washed, and fresh medium containing estrogen was added. Cells were harvested 24 h afterward and assayed for LUC activity using the Luciferase Reporter Assay system (Promega). LUC activity was calculated as light units/unit protein, which yields values similar to those based on internal β-galactosidase transfection standards (24). Data are presented as the means of at least three determinations, and all experiments were repeated at least twice with similar results.

RNA Isolation and Semiquantitative Reverse-Transcription PCR Analysis. RNA was isolated with TRIzol (LifeTechnologies) according to the manufacturer’s instructions and was quantitated by UV absorption. Deoxythymidylic acid oligomer (oligo dT)-primed reverse transcription was performed using 1 μg of total RNA in a final volume of 20 μl, and the resulting cDNA was further diluted to 100 μl. Comparative semiquantitative PCR was performed as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was first amplified at low-cycle number (GAPDH primer sequences: sense, CCACCCAATGGAATCATCCATGGCA; antisense, TCTAGACGCAGGTCAGGTCCACC). The resulting 600-bp products were visualized by ethidium bromide staining and then excised from the gel and reamplified to obtain similar intensities for GAPDH signals with primer sequences: sense, −147, −362, −380, and 1660 bp relative to the transcription initiation site (Fig. 1, A and B). We isolated the portion of HPSE regulatory region (−1791/+109) that contains the four identified estrogen response elements and introduced it in front of the LUC reporter gene. This 1.9-kb region conferred transcription promoter activity, enabling LUC expression in breast, prostate, and bladder carcinoma cell lines transiently transfected with the reporter construct (~500 fold increase versus cells transfected with basic plasmid alone, not shown). To examine the responsiveness of the slow-release estrogen pellet (Innovative Research of America, Sarasota, FL) implanted s.c. The ovariectomy and implantation of the pellets were performed 4 days earlier. Matrigel plugs were removed 7 days postimplantation, photographed, fixed in 4% paraformaldehyde, and processed for histological examination and immunostaining. For vessel density analysis, 5-μm sections of paraffin-embedded Matrigel plugs were stained with Masson-Trichrom. The microvessel density was counted by a blinded observer on a ×200 microscopic field as described (26). A total of 30 fields/condition (6 plugs × 5 fields) were analyzed, and the mean value ± SE was determined.

Immunohistochemistry. Immunohistochemistry was performed as described with minor modifications (12, 14). Briefly, 5-μm sections of paraffin-embedded Matrigel plugs were deparaffinized and rehydrated. Tissue was then incubated in 3% H₂O₂, denatured by boiling (3 min) in a microwave oven in citrate buffer (0.01 M, pH 6.0), and blocked with 10% goat serum in PBS. Sections were incubated with a rabbit antihuman heparanase antibody diluted in 10% goat serum in PBS, or with 10% goat serum alone as control, followed by incubation with horseradish peroxidase-conjugated antirabbit antibody (Dako Corporation, Carpinteria, CA). The heparanase antibody was raised against a peptide 273KTAKMLKSFKLAKGEV1206 located in the 50-kDa unit of the active heparanase enzyme and kindly provided by Dr. R. L. Heinrikson (Pfizer, MI; Ref. 27). Color was developed by using the Zymed AEC substrate kit (Zymed Laboratories, South San Francisco, CA) for 10 min, followed by counter staining with Mayer’s hematoxylin.

RESULTS

The Heparanase Gene Regulatory Region Contains Four Sequences Homologous to Consensus Estrogen Response Element and Is Responsive to Physiological Concentrations of Estrogen. Computerized analysis of the heparanase gene 3.5-kb regulatory region (28) revealed four putative estrogen response element sequences (minimum 70% homology to the consensus estrogen response element), located at positions −147, −362, −740 and −1660 bp relative to the transcription initiation site (Fig. 1, A and B). We isolated the portion of HPSE regulatory region (−1791/+109) that contains the four identified estrogen response elements and introduced it in front of the LUC reporter gene. This 1.9-kb region conferred transcription promoter activity, enabling LUC expression in breast, prostate, and bladder carcinoma cell lines transiently transfected with the reporter construct (~500 fold increase versus cells transfected with basic plasmid alone, not shown). To examine the responsiveness of the

Fig. 1. Schematic representation of heparanase (HPSE) gene regulatory sequence and regions of homology to the consensus estrogen response element (cERE). A, red arrows indicate regions with 70–80% homology to the cERE. The bent arrow represents the transcriptional initiation site. B, sequences in the HPSE promoter region homologous to cERE and their location.

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**Fig. 2.** Estrogen increases heparanase promoter activity in breast cancer MCF-7 cells. MCF-7 cells were transiently transfected with a luciferase (LUC) reporter gene driven by the heparanase promoter. After overnight recovery, cells were treated with either different concentrations of estrogen, or vehicle alone, as described in “Materials and Methods.” Luciferase activity was determined 24 h later. Results are normalized for equal protein content and expressed as a percentage of control (vehicle alone). Note that the maximal response was obtained at physiological concentrations of estrogen.

**Fig. 3.** Effects of estrogen and its antagonist on heparanase (Hpa = HPSE) mRNA expression in breast cancer cell lines. MCF-7 cells were treated (8 h) with either 1 × 10−7 M estrogen (E), in the absence or presence of 1 × 10−7 M pure estrogen antagonist ICI 182,780 (ICI), or 5 × 10−6 M tamoxifen (TAM). RNA was then isolated from the cells and comparative semiquantitative PCR was performed. Aliquots (10 µl) of the PCR products were separated by 1.5% agarose gel electrophoresis and visualized (bottom). The intensity of each band was quantitated using an EagleEye system, and the results are expressed as band intensity relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; top). In MCF-7 cells, estrogen treatment resulted in an ~400% increase in heparanase mRNA levels. Similar results were obtained with the T47D cells (not shown). The pure estrogen antagonist ICI 182,780 completely abolished the effect of estrogen on heparanase gene expression. Treatment of the estrogen receptor-negative MDA-MB-231 cells with estrogen did not cause any change in heparanase mRNA levels (inset).

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heparanase promoter to estrogen, estrogen receptor-positive MCF-7 breast carcinoma cells were transiently transfected with the above described reporter construct [HPSE (−1791/+109)-LUC] and further treated with estrogen at concentrations ranging from 1 × 10−9 to 1 × 10−6 M, as described in “Materials and Methods.” As shown in Fig. 2, the heparanase promoter region exhibited maximal hormonal inducibility at 1 nM estrogen, yielding a 2.7-fold increase (P < 0.0004), as compared with untreated cells. When the same construct was transfected into MDA-MB-231 breast cancer cells, lacking the estrogen receptor, estrogen treatment did not result in any change in LUC activity (not shown).

**Induction of Heparanase mRNA Expression through Estrogen Receptor-Dependent Mechanism.** Next, we studied the estrogen regulation of heparanase mRNA expression by various human breast cancer cell lines, using semiquantitative reverse-transcription PCR. Confirming the promoter data, estrogen treatment induced a marked increase in heparanase mRNA levels produced by estrogen receptor-positive human breast carcinoma cells (MCF-7, T47D), but did not affect heparanase expression in estrogen receptor-negative MDA-MB-231 cells (Fig. 3). We tested several doses of estrogen, representing the hormone levels through menarche (10−10−10−9 m) and pregnancy (10−8 m), and supra-physiological concentrations (10−7−10−6 M). The response to estrogen occurred in a manner typical for estrogen-mediated bioeffects (29): a significant increase in the level of heparanase mRNA was observed at concentrations of estrogen ranging from 10−8−10−10 m, and the maximum effect was exerted at 1 nM estrogen (400% increase, Fig. 3 and data not shown). This effect was time-dependent, first occurring within 4 h, reaching a maximum at 8 h (not shown). To distinguish between direct transcriptional effects through the classic estrogen receptor pathway and a number of recently described alternative mechanisms of estrogen regulated responses (30), we investigated the effect of the pure estrogen receptor antagonist ICI 182,780 (31) on estrogen-mediated induction of heparanase mRNA. The presence of 1 × 10−7 m ICI 182,780 completely abolished the effects of estrogen on heparanase mRNA expression by MCF-7 cells (Fig. 3). The partial estrogen receptor antagonist tamoxifen, the most widely used drug in endocrine therapy of breast cancer, exerted inhibitory effects on estrogen-induced heparanase expression only at a concentration as high as 5 × 10−6 M (Fig. 3). This concentration is 15 times higher than that observed in the serum of 4-hydroxy-tamoxifen-treated breast cancer patients (32) or that required to inhibit proliferative effects of estrogen on breast carcinoma cells.

**Estrogen Induces Heparanase Protein Expression in Vivo, Associated with an Increase in Breast Tumor Angiogenesis.** Estrogen receptor-positive MCF-7 breast carcinoma cells were mixed with Matrigel (reconstituted basement membrane preparation; Ref. 33) and injected s.c. into ovariectomized nude mice (implanted with either placebo or slow release estrogen pellets before cell injection). Upon injection, the liquid Matrigel rapidly forms a solid gel plug that serves as a supporting medium for the breast carcinoma cells. Similar to intact basement membrane, Matrigel contains HS as a major polysaccharide component, along with characteristic ECM proteins. Matrigel also contains basic fibroblast growth factor and other important angiogenic and growth-promoting factors that are found in native ECM (34). Hence, the Matrigel in this experimental system serves not merely as an inert vehicle for the embedded cells, but also as an agent maintaining the natural interactions existing between breast tumor cells and the adjacent ECM, providing, among other effects, a source of basement membrane-sequestered, HS-bound angiogenic factors, which could be released by heparanase. Heparanase has been previously shown to elicit an angiogenic effect in the Matrigel plug assay, degrading HS and releasing these angiogenic factors (21).

As shown in Fig. 4, high levels of heparanase protein were detected in Matrigel-embedded MCF-7 cells injected into mice implanted with estrogen pellets (Fig. 4F), as compared with low levels of heparanase observed in MCF-7 cells injected into mice with placebo pellet (Fig. 4E). The increase in heparanase expression correlated with the pronounced angiogenic response induced by Matrigel-embedded MCF-7 cells in the presence of estrogen. Little or no neovascularization was observed with MCF-7 cells in the absence of estrogen. The angiogenic response was reflected by a network of capillary blood vessels in the Matrigel plugs excised...
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Fig. 4. Estrogen induces heparanase protein expression and tumor angiogenesis. MCF-7 cells were mixed with Matrigel and injected s.c. into ovariectomized nude mice, which were first implanted with either placebo or slow release estrogen pellets. Matrigel plugs were excised 6 days later. A and B, representative plugs; C and D, Masson-Trichrom staining (magnification, ×320); E and F, immunohistostaining with antiheparanase antibodies of plug sections from mice implanted with the placebo (A, C, and E) or the estrogen slow-release pellet (B, D, and F; magnification, ×200). White arrowheads, MCF-7 cells embedded in Matrigel; black arrows, newly formed blood vessels.

from the estrogen-treated mice (Fig. 4, B and D) versus little or no vascular response in placebo-treated mice (Fig. 4, A and C) and by a respective difference in microvessel density determined in the isolated Matrigel plugs excised from each group. In the presence of estrogen, the microvessel count was 24.2 ± 10.7 vessels/microscopic field, whereas the corresponding value in the absence of estrogen was 14.6 ± 8.3 vessels/field; *P < 0.0001* Student’s *t* test. These results suggest that in breast carcinoma *in vivo* estrogen induces an increase in heparanase protein levels, and this increase may be responsible for augmented tumor neovascularization.

**DISCUSSION**

The heparanase gene regulatory sequence has been reported previously (28), and transcription factors (*i.e.*, Sp1, Ets), as well as epigenetic changes (*i.e.*, methylation), affecting *HPSE* promoter activity are being characterized (28, 35–37). Yet, the molecular pathways responsible for the enhanced *HPSE* expression in developing tumors, as compared with normal tissues of the same origin (13–16, 22, 23) remain poorly understood. We have demonstrated estrogen responsiveness of the heparanase gene promoter and have found that exposure to physiological concentrations of estrogen induces heparanase expression in breast carcinoma cells. The estrogen effect on heparanase expression occurred in estrogen receptor-positive, but not in estrogen receptor-negative breast carcinoma cell lines, and was inhibited by the antiestrogen ICI 182,780, known to block responses mediated through estrogen receptor signaling (31). Tamoxifen, the antiestrogen conventionally used in endocrine therapy of breast cancer, also inhibited estrogen-induced heparanase expression, although at concentrations significantly exceeding those measured in the blood of tamoxifen-treated patients (32). These data indicate that the classic estrogen receptor pathway is involved in transcriptional activation of heparanase, rather than numerous alternative mechanisms of estrogen regulated cell responses (30). It is expected that further refined analysis of *HPSE* regulatory sequence, applying truncated reporter constructs and chromatin immunoprecipitation method, will enable the location of the precise binding site(s) of the estrogen receptor complex. Recently, it has been reported that Ets and Sp1, previously shown to regulate *HPSE* gene expression (35, 36), may mediate estrogen cellular effects, particularly on vascular endothelial cells (30, 38, 39). This raises the possibility that the observed estrogen stimulation of heparanase expression in breast cancers may be attributable, in part, to interplay between estrogen receptor complex and Ets or Sp1 transcription factors.

Estrogen has been implicated in breast tumorigenesis primarily via its stimulation of cell proliferation, and several cell-cycle regulatory proteins have been identified as estrogen target genes that mediate its cancer-promoting effect (1–5). However, in addition to enhanced cell proliferation, imbalanced interactions of tumor cells with the host microenvironment are currently viewed as a fundamental feature of neoplasia (6, 7). Enzymatic remodeling and degradation of ECM and, in particular of epithelial and subendothelial basement membranes, represent a universal mechanism of such interactions and a prerequisite for tumor progression (reviewed in (6)). Heparanase is the predominant enzyme that cleaves HS in the ECM, basement membrane and cell surface (13–16). HS interacts, through specific attachment sites, with the structural proteins (*i.e.*, collagen, laminin, fibronectin) of the ECM and, hence, play an essential role in maintaining the insolubility and barrier properties of the ECM and basement membrane (12, 14, 18, 19). HS moieties in the ECM are also known to specifically bind various members of the heparin-binding growth factor family, serving as their extracellular reservoir (39). Many of the heparin-binding growth factors (*i.e.*, basic fibroblast growth factor, vascular endothelial growth factor) sequestered by HS in the ECM are implicated in tumor growth and in angiogenesis (40). The previously demonstrated role of heparanase in cancer development and in progression has, therefore, been attributed to disassembly of extracellular barriers for tumor cell invasion (*i.e.*, ECM, basement membranes) and release of HS-bound bioactive molecules from ECM depots (12–14, 17, 21, 22, 40, 41). In human mammary carcinoma, heparanase mRNA and protein are highly expressed in both the *in situ* and invasive components of ductal and lobular origin, whereas normal breast tissue expresses little or no heparanase (12). Heparanase expression correlates with both aggressiveness of human breast carcinoma cell lines (14) and the metastatic potential of breast tumors (23). Elevated levels of heparanase have been found in body fluids of patients with active breast cancer disease versus healthy donors (40). Moreover, stable overexpression of heparanase in MCF-7 cells, inoculated into nude mice,
resulted in a significantly greater tumor vascularization and a 4-fold increase in tumor size (unpublished results), emphasizing a casual involvement of heparanase in primary breast tumor take and angiogenesis. These data, together with our report here on estrogen inducibility of the heparanase gene and the fact that the majority of primary breast tumors are estrogen receptor positive (41), suggest that in breast tumorigenesis heparanase may serve as a novel downstream estrogen effector that regulates tumor-stromal interactions, in addition to its documented effects on cell proliferation, which have been reported for previously described estrogen target genes. The observed augmented angiogenesis, exerted by MCF-7 cells after estrogen-induced increase in heparanase levels (Fig. 4), supports this proposed mode of action.

Most effectively, estrogen induced heparanase expression at low physiological concentrations (1 \times 10^{-9} M), typical of the menstrual cycle, but was ineffective at higher doses, representing an estrogen level consistent with pregnancy or at supra-physiological concentrations (1 \times 10^{-8}-1 \times 10^{-6} M). Both the early menarche and late first pregnancy have been identified as risk factors for breast cancer. It was, therefore, suggested that the continuous exposure to low (menarche) estrogen levels, rather than to high concentrations of estrogen during pregnancy, is responsible for breast tumor stimulation (1). The observed inducibility of heparanase expression by low estrogen levels and, on the other hand, the lack of an estrogen effect on heparanase at higher concentrations are in agreement with the above-mentioned interpretation of epidemiological data (1). It is conceivable that heparanase expression induced in breast epithelium by low physiological levels of estrogen contributes, among other factors, to primary breast tumor growth and neovascularization via previously described molecular mechanisms (i.e., breakdown of extracellular barriers and release of HS-bound angiogenic and growth promoting factors). This effect could be particularly important at the initial stages of breast tumorigenesis, when >70% of all tumors are reportedly estrogen-receptor positive (42). On the other hand, estrogen receptor-negative breast tumors, estrogen-independent, increased expression (14) of heparanase has been found and may account, at least in part, for the more aggressive metastatic phenotype, poor prognosis, and failure of antiestrogen therapy. In this type of tumor, administration of specific heparanase inhibitors, which are currently under development (43, 44), might be considered in the future as an appropriate therapeutic modality.

REFERENCES


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