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Expression of the cyclin kinase inhibitor, $p27^{kip1}$, in developing and mature human kidney

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Expression of the cyclin kinase inhibitor, $p27^{kip1}$, in developing and mature human kidney. It has been shown that glomerular visceral epithelial cells (VEC) proliferate during glomerulogenesis, but differentiated VEC of the fetal kidney do not. It is also recognized that the proliferative capacity of the VEC in mature kidneys is very limited, and, according to some investigators, may be completely absent. The basis for this remains unknown. Cell proliferation is controlled by cell cycle-related proteins, of which one class, the cyclin kinase inhibitors (CKI), cause cell cycle arrest and inhibit proliferation. A role for CKI in kidney development is not known. Accordingly, we examined the expression of the CKI $p27^{kip1}$ (p27) in developing and mature human kidney tissue. Concomitant expression of markers of cell proliferation, Ki-67-related antigen (Ki-67) and proliferating cell nuclear antigen (PCNA), also were examined in fetal and mature human kidney tissue by immunocytochemical techniques. In developing kidney, Ki-67 and PCNA expression are most pronounced in the nephrogenic zone where expression correlates inversely with increasing glomerular maturation. In well-differentiated glomeruli, Ki-67 and PCNA expression is present in some parietal epithelial cells but is absent in the VEC. In contrast, p27 staining exhibits a reverse gradient of expression. p27 is absent in the proliferating tissue exhibiting the earliest stages of differentiation, whereas expression is widespread in the differentiated epithelial cells of more mature glomeruli, in which detectable cell proliferation has ceased. Expression of p27 was not identified in fetal mesangial or glomerular endothelial cells. In the mature human kidney, the pattern of p27 expression identified in differentiated fetal glomeruli persists and appears to be constitutive and specific for glomerular VEC. This pattern of p27 expression in terminally differentiated VEC may explain their limited proliferative capacity in response to injury. This is the first demonstration of a potential role for p27 in human renal development.

The capacity of the glomerular visceral epithelial cell (VEC) or podocyte to undergo cell proliferation is a question of great current interest. There are studies in human and experimental animals that suggest that these cells have the capacity to enter the cell cycle by virtue of their ability to incorporate tritiated thymidine [1], by the demonstrated expression of the cell cycle-associated proteins proliferating cell nuclear antigen (PCNA) and Ki-67-related antigen (Ki-67) in these cells [2–4], and by the recognition of mitotic figures in these cells at sites of apparent epithelial cell prominence or possibly increased cell number [2, 5]. Other studies suggest that VEC may lack the capacity to undergo complete cell division [reviewed in 6, 7]. While acknowledging that these cells may traverse early steps through the cell cycle, some investigators suggest that VEC lack the ability to undergo full cytokinesis/cell division, and as evidence demonstrate the presence of multinucleated podocytes and an inability to demonstrate increased cell numbers after a variety of injuries have been administered to these cells [8–11]. Studies in experimental models of VEC injury indicate that the inability of VEC to undergo adequate proliferation following injury, in conjunction with detachment from the glomerular capillary basement membranes, is a critical step in the development of the lesion of focal and segmental glomerular sclerosis [6–8, 11].

Cell proliferation is controlled by cell cycle regulatory proteins. Progression through the cell cycle requires that cyclin dependent kinases (CDK) be activated by cyclins [12]. Two families of cyclin kinase inhibitors (CKI) inactivate target CDK's, thereby arresting the cell cycle and inhibiting proliferation. The CKI p27 inhibits cyclin-CDK complexes in both G1 and S phase of the cell cycle. We have shown that mesangial cell proliferation is determined by the levels of p27 [13], but its role in the VEC remains to be fully elucidated.

Glomerulogenesis in the developing fetal metanephric kidney affords an excellent opportunity to examine how specific cell cycle regulatory proteins may participate in the control of cell proliferation. A well-characterized sequence of events, beginning with a stage of widespread, even uniform, proliferation of the immature epithelial structures differentiating from metanephric blastema, culminates in differentiated glomeruli, where proliferation of VEC essentially ceases [14, 15]. We therefore examined how the expression of p27 in developing metanephric human kidney tissues might be reflected in these established patterns of cell proliferation. Our studies demonstrate strong and uniform expression of p27 in differentiated glomerular VEC. The *de novo* expression of p27 corresponds to the stage of development where proliferation of differentiating epithelium ceases and differentiation into the specific podocyte phenotype occurs. This specific pattern of p27 in fetal VEC, in contrast with all other glomerular cell types examined in the developing kidney, persists into adulthood. The localization of p27 to this highly differentiated cell type may explain, at least in part, the low proliferative capacity of the mature VEC.

[1] See *Editorial* by Wolf and Neilson, p. 1087

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METHODS

Source of tissue

Human fetal kidneys with estimated gestational age ranging from 54 to 104 days ($N = 26$) were obtained fresh from tissue examined after therapeutic abortions. Eleven of the samples (estimated gestational age ranging from 56 to 81 days) were fixed in 10% neutral buffered formalin and then processed and embedded in paraffin according to conventional techniques. Fifteen kidneys (estimated gestational ranging from 54 to 105 days) were fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid) and processed and embedded in paraffin. Mature human kidney was obtained from fresh tissue examined after nephrectomy ($N = 17$). All of these samples were fixed in 10% neutral buffered formalin and processed and embedded in paraffin.

Antibodies

$p27^{kip1}$ (p27). p27 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) is an affinity-purified goat polyclonal antibody raised against a peptide corresponding to amino acids 181-298 mapping at the carboxy terminus of p27 of human origin. Its specific recognition of p27 has been demonstrated previously by both Western blotting [16] and immunoprecipitation [17] techniques.

Ki-67 related antigen (Ki-67). MIB-1 (Coulter/Immunotech, Miami, FL, USA) is a purified murine monoclonal IgG antibody raised against a peptide corresponding to a 1002 base pair sequence of Ki-67-related antigen cDNA [18]. Specificity of this antibody to Ki-67 antigen, a human nuclear cell proliferation-associated antigen, has been demonstrated by Western blotting [18, 19] and immunohistochemistry [18, 20].

PCNA. A murine monoclonal antibody to PCNA/cyclin, 19A2 (Coulter Corp., Hialeah, FL, USA) has been well characterized and was used in methyl Carnoys' fixed tissues as previously described [21, 22].

Immunohistochemistry

Formalin-fixed tissue sections (used for immunohistochemistry studies of p27 and Ki-67) were deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with hydrogen peroxide and the samples were then rinsed in PBS. To obtain an adequate signal with the p27 antibody, the slides were heated for 30 minutes in a pressure cooker in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA). To reduce background, the sections to be stained for p27 were blocked for 30 minutes in 10% normal rabbit serum. The sections were then incubated with p27 or MIB-1 primary antibody diluted in PBS containing 1% bovine serum albumin for one hour at room temperature. After PBS washes, the tissue was incubated sequentially with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC-Elite; Vector Laboratories) and then 3,3′-diaminobenzidine (with nickel chloride enhancement) to give a black reaction product. The tissue sections were counterstained with methyl green, dehydrated and coverslipped.

Methyl Carnoy's fixed tissue sections (used for immunohistochemistry studies of PCNA) were deparaffinized, rehydrated, blocked with hydrogen peroxide, and washed with phosphate buffered saline (PBS). The tissue sections were then incubated with anti-PCNA antibody and processed as above. Controls for all of the immunocytochemistry included substitution of the primary antibody with normal IgG from the same species as the primary antibody at a similar concentration.

RESULTS

Fetal kidney tissue was obtained with gestational ages ranging from 54 to 105 days. The tissue sample exhibited age-appropriate structures, including undifferentiated blastema and early glomerular vesicles at the outer margin of the cortex, comma and S-stage glomeruli beneath the outer cortex, and more mature glomeruli in the inner cortex.

Immunohistochemistry

Ki-67. Expression of the proliferation marker Ki-67 was most pronounced in the nephrogenic zone in the first stages of glomerular epithelial differentiation, where many cells in differentiating vesicles and folding, developing glomeruli ("comma" shaped glomeruli) were actively replicating (Fig. 1A). The S-shaped glomeruli exhibited varying expression of Ki-67, but generally there was decreasing Ki-67 expression corresponding to a progressively greater glomerular maturation. In well-differentiated glomeruli, Ki-67 expression was uniformly undetectable in VEC (Fig. 1B). At the stage of full glomerular differentiation, scattered non-uniform Ki-67 staining was present in some parietal epithelial cells and within the glomerular tuft in locations corresponding to expression by mesangial or endothelial cells. More specific identification of the scattered, occasionally positive cells was not achieved by histologic examination.

PCNA. As described and illustrated previously [14], PCNA expression was most heavily concentrated in the nephrogenic zone with a declining gradient as glomerular maturation increased (Fig. 1). The pattern of Ki-67 antigen and PCNA expression were essentially indistinguishable within glomeruli at all stages of differentiation (Fig. 1A, 1G).

p27. Staining for the CKI p27 in fetal tissue exhibited a gradient of expression with absence in the least differentiated structures to most abundant staining in fully differentiated glomeruli (Fig. 1 C, D). The undifferentiated metanephric blastema, ureteric buds, and early glomerular vesicles of the subcapsular cortex showed no expression of p27. As folding of the vesicular structures commenced ("comma shaped" glomeruli), focal and weakly detectable p27 expression was identified in individual cells, and this expression extended to a greater proportion of the epithelial cells in conjunction with further differentiation to the phase of S-shaped glomeruli (Fig. 1 C, E). When the glomerulus is fully differentiated, VEC uniformly exhibit p27 expression with strong immunohistochemical staining (Fig. 1 D, F). No expression is identified in mesangial or endothelial cells. Thus, staining for p27 and markers of DNA synthesis (PCNA, Ki-67) did not co-localize. In adult kidney tissue, p27 expression was found constitutively and generally uniformly in glomerular visceral epithelium and in some parietal epithelial cells (Fig. 1H). In contrast, mesangial and endothelial cells did not exhibit detectable p27 expression.

DISCUSSION

Our study is the first demonstration of the expression of one class of cell cycle regulatory proteins, CKI, in human kidney. We show that constitutive expression of the CKI p27 is restricted to differentiated glomerular VEC, and that the *de novo* expression of p27 corresponds with the transition of the proliferating immature VEC to mature, non-proliferating VEC.

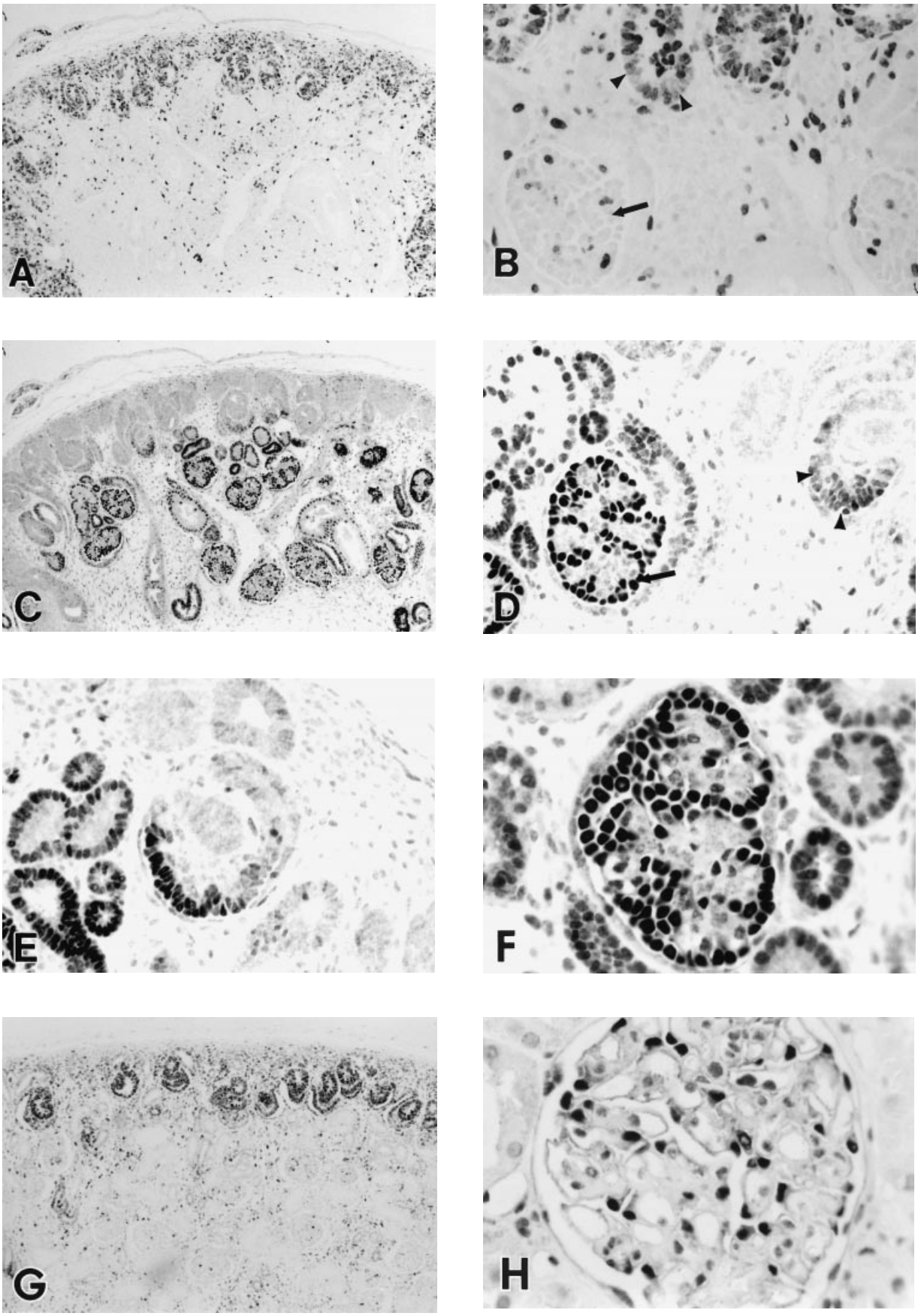
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Fig. 1. (*A*.) Expression of the cell proliferation marker, Ki-67-related antigen, in human fetal kidney of 67 days gestation. There is widespread proliferation of cells, indicated by dark staining nuclei, in a zone of nephrogenesis immediately under the renal capsule, which involves blastemal cells, vesicles, and folding epithelial structures corresponding to the earliest stages of glomerulogenesis. The zone of cell proliferation is sharply demarcated, with only scattered proliferating cells present in the remaining portions of the renal parenchyma. Expression of PCNA was indistinguishable from that illustrated here. (*B*.) Higher power view of kidney in A showing widespread expression of Ki-67-related antigen in tubular structures in regions closest to the renal capsule (top), with scattered expression of this antigen in more differentiated renal cells (bottom of photo) that is not linked to any specific differentiating cell type. There is a transition (arrowheads) where folding, differentiating glomeruli exhibit an intermediate pattern of expression of Ki-67-related antigen indicating some, but not all, of the cells in these structures are still engaged in cell cycle traverse. A more differentiated glomerulus (arrow) is notable for absence of clearly detectable Ki-67-related antigen expression in the great majority of cells present, including visceral epithelial cells. (*C*.) Expression of p27 in a sequential tissue section from the same kidney as A and B. Expression of p27, indicated by darkly stained nuclei, is largely confined to the VEC of differentiating glomeruli, and is absolutely distinct from the pattern of cell proliferation demonstrated in A. (*D*.) Higher power view of kidney in C, showing a field matching that illustrated in B, stained to demonstrate expression of p27. The early differentiating epithelial structures do not express p27, while differentiated glomerular VEC do (arrows). Again, the point of transition where cells cease to express markers of proliferation, as illustrated in B, marks the earliest stage of glomerulogenesis where p27 expression can be identified (arrowheads). (*E*.) Example of the earliest stage at which p27 first becomes detectable in the differentiating epithelium of a folding, developing glomerulus. The metanephric blastema and great majority of interstitial cells do not express this molecule. p27 expression is detectable in some tubular structures, also evidenced in Figure 1C and D. (*F*.) p27 is uniformly expressed by differentiated VEC in developing glomeruli. (*G*.) Expression of PCNA in a human fetal kidney of 73 days gestation. The pattern of staining is indistinguishable from that of Ki-67 (Fig. 1A), although the immunohistochemical stains achieved with this antibody are less intense than those obtained with the Ki-67 antibody. (*H*.) Constitutive expression of p27 in VEC, but not other cell types, of adult human kidney. There is persistence of the pattern of expression identified in differentiated fetal glomeruli, as illustrated in Figure 1F.

There are several lines of evidence to show that the CKI p27 controls cell proliferation. The overexpression of p27 in cultured cells causes cell cycle arrest, whereas lowering p27 levels increases proliferation both in renal (mesangial) and non-renal cells in culture [13, 24]. Recent studies have demonstrated that p27 expression may be an important determinant of renal cell proliferation in several *in vivo* models of renal disease. In the Thy 1 model of mesangial proliferative glomerulonephritis, the onset of mesangial cell proliferation coincides with a decrease in p27 levels and cessation of proliferation coincides with increasing p27 levels as they return to normal patterns [25]. More recently, we have shown that injury to the VEC in the passive Heymann nephritis model of membranous nephritis is associated with increased levels of this CKI [26]. Finally, it has also been shown that p27 knockout mice exhibit widespread organ hypercellularity, but are otherwise architecturally normal, indicative of a generalized defect in regulation of cell number. This is presumed to be the result of altered regulation of cell proliferation [27]. The p27 knockout mouse specifically exhibits normal architectural organization of the glomerulus as assessed histologically and by ultrastructural strudies (C.E. Alpers, personal observations).

The present study extends these previous studies of p27 in cultured cells and in experimental models of renal disease to human kidneys and correlates p27 levels with defined patterns of cell proliferation. In this study, we demonstrated patterns of expression of Ki-67 and PCNA, both markers of cell proliferation, at stages of glomerulogenesis (vesicular transformation of epithelial cell progenitors from the metanephric blastema, enlargement of the epithelial cell mass through the folding stages–comma and S-shape–of early glomerular development) previously demonstrated to have features of extensive cell proliferation [14, 23]. There is decreasing expression of these markers of proliferation with terminal differentiation of the glomerulus. The absence of these cell proliferation markers in mature VEC supports the concept that these cells do not, in general, replicate after terminal differentiation [2, 5].

The developing kidney, with these defined stages of cell proliferation, is therefore an excellent organ to evaluate the possibility that p27 is important in determining the replicative capacity of specific renal cell types and the VEC in particular. This study found that p27 is first detectable at that stage in glomerulogenesis when cell proliferation diminishes, and its uniform expression occurs in a cell type long recognized to have the least proliferative potential of any renal parenchymal cell [3]. Together, these findings provide substantial support for the concept that p27 is an important negative regulatory molecule for VEC replication. The persistent and constitutive expression of p27 in the normal adult kidney offers one mechanism to explain the limited capacity for replication of these cells. It does not, however, explain the failure to complete cytokinesis by these cells that is observed in some experimental settings [7, 11], since this process occurs later in the cell cycle than the G_1 to S transition regulated by p27.

The finding that p27 was not expressed in undifferentiated blastemal cells, while increasing in conjunction with the acquisition of podocyte phenotype, is consistent with the possibility that this CKI also may function to promote and/or maintain cell differentiation in some cell types, independent of its defined role in regulation of cell proliferation [28]. This possibility is strengthened by p27's continued expression in mature VEC. The factors that regulate this p27 expression in VEC are not known. In non-renal cells, it is known that p27 is post-translationally modified by the ubiquitin—proteasome pathway [29]. It remains to be established if this pathway is also relevant to renal cell types.

VEC injury is central in many forms of human renal disease, including glomerulonephritis, membranous nephropathy minimal change disease, and collapsing and HIV-associated nephropathy. It can be hypothesized that if p27 expression is not downregulated, as exemplified during the early proliferative stages of glomerulogenesis, the proliferative capacity of the VEC remains in check even in the face of tissue injury. Such a scenario could then lead to a progressive injury process similar to that identified in the partial ablation [9], ablation/hypertension [10], and Masugi nephritis [29] models in rodents whereby injured VEC, detached from the capillary basement membrane and unable to undergo replication, result in leaving a denuded glomerular basement membrane. This in turn would contribute to protein leak into the urinary space, synechial attachment to Bowman's capsule, and eventually to segmental sclerosis. Alternately, it can be hypothesized that if p27 expression can be downregulated under certain conditions, it might help to explain the accumulation of epithelial

cells that is a feature of some diseases such as collapsing and HIV-associated nephropathy. It will be important to examine multiple examples of glomerular diseases associated with VEC injury for expression of relevant cell cycle regulatory proteins in order to test this hypothetical sequence of injury.

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APPENDIX

Abbreviations used in this article are: ABC, avidin-biotin-peroxidase complex; CDK, cyclin dependent kinases; CKI, cyclin kinase inhibitors; KI-67, Ki-67-related antigen; p27, cyclin kinase inhibitor $p27^{kip1}$, PCNA, proliferating cell nuclear antigen; VEC, visceral epithelial cells.

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