Overexpression of Cyclins D1 and E Is Frequent in Bronchial Preneoplasia and Precedes Squamous Cell Carcinoma Development

Fulvio Lonardo,1,2 Valerie Rusch,3 John Langenfeld,3 Ethan Dmitrovsky,4 and David S. Klimstra5

Laboratory of Molecular Medicine, Department of Medicine (F. L., V. R., J. L., E. D.); Department of Pathology (F. L., D. S. K.); Thoracic Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York 10021.

ABSTRACT

Increased protein expression of the G1 cyclins D1 and E is reported in invasive non-small-cell lung carcinoma. However, during transformation of the bronchial epithelium, overexpression of these species occurs, and their relationship to aberrant expression of p53 and retinoblastoma (Rb) has not been described previously. To determine the expression of these cell cycle regulators during the development of invasive squamous cell carcinoma (SCC) of the lung, the immunohistochemical expression patterns in normal bronchial epithelium (n = 36), squamous metaplasia (SM); n = 28), and epithelial atypia (n = 34) were compared with that in low-grade dysplasia (LGD); n = 17), high-grade bronchial dysplasia (HGD); n = 30), and SCC (n = 36). Monoclonal anti-p53 Pab1801, polyclonal anti-cyclin D1 DC56, monoclonal anti-cyclin E HE12, and monoclonal anti-Rb OP-66 antibodies were used. Cyclin D1 was not expressed in normal bronchial epithelium but was detected in 7% of SMs, 15% of atypias; 18% of LGDs, 47% of HGDs, and 42% of SCCs. Cyclin E was not detected in normal epithelium (n = 24), SM (n = 16), or LGD (n = 12), but it was found in 9% of atypias (2 of 22), 33% of HGDs (7 of 21), and 54% of SCCs (13 of 24). p53 was not expressed in normal epithelium, SM, and LGD, but it was overexpressed in 6% of atypias, 53% of HGDs, and 61% of SCCs. Abnormal Rb expression was found only in 2 of 36 cases of SCC. A total of 91% of HGDs and 92% of SCCs exhibited overexpression of at least one of the p53, cyclin D1, or cyclin E species. However, no link was observed between overexpression of p53 and the overexpressed G1 cyclins in preneoplastic lesions. Overexpression of cyclin D1, cyclin E, and p53 occurs frequently and independently in pulmonary SCC and is detected in lesions before the development of invasive carcinoma. In contrast, altered Rb expression is a late and infrequent event in squamous cell carcinogenesis.

INTRODUCTION

The G1 cyclins, cyclin D1 and cyclin E, and the tumor suppressor genes p53 and Rb6 are key regulators of the cell cycle (1–3). Rb provides a check point in the G1 to S-phase transition; its phosphorylation allows the transcription of factors required for DNA replication. The phosphorylation of Rb occurs through complexes including cyclin D/CDK4 and cyclin E/CDK2, which are inhibited by CDK inhibitors including p15, p16, and p21 (1–3). Alterations of p53, cyclin D1, and Rb have been described in invasive lung cancer. Overexpression of p53 is reported in 57–87% of SCCs (5–7). Cyclin D1 gene amplification is reported in 13–15% of NSCLCs, predominantly in SCCs and large cell carcinomas (8), and >40% of NSCLCs are reported to be positive for cyclin D1 by IHC (9, 10). The reported frequency of aberrant expression of Rb ranges from 15–34% in NSCLC (11, 12) and from 17–38% in SCC (10, 11). In contrast, in small cell carcinoma of the lung, aberrant Rb expression is almost universally detected (13, 14). Only one preliminary report of cyclin E overexpression in NSCLC exists to date (15). However, in vitro studies using carcinogen-induced transformation of immortalized human bronchial epithelial cells suggest that cyclin E plays a key role in the transformation of bronchial epithelial cells (16).

Pathological and epidemiological studies (20–22) as well as animal models (23) support the view that SCC arises through progressive alterations of the bronchial epithelium, starting with SM and progressing through increasing degrees of dysplasia to CIS. However, the molecular events that underlie this histological progression are not well understood. Chromosomal losses involving 3p, 9p, and 5q are reported in bronchial precursor lesions (24–27), as is overexpression of p53 (7, 28–30) and the epidermal growth factor receptor (7). However, the incidence and timing of alterations in cyclin D1 and cyclin E in association with aberrant p53 and Rb expression have not been studied previously. The current study examines preneoplastic bronchial lesions and their corresponding SCCs to determine the patterns of expression and coexpression of these important cell cycle regulator genes and how these may be linked to the development of overt cancer.

MATERIALS AND METHODS

Lung specimens from resections performed for SCC that also contained anatomically distinct areas of bronchial atypia, SM, dysplasia, or CIS were identified retrospectively from the surgical pathology files at the Memorial Sloan-Kettering Cancer Center. Bronchial mucosa was diagnosed as atypical when an increase in thickness and mild cytological atypia were noted, but the uppermost layer of ciliated cells was preserved. SM was diagnosed when the bronchial epithelium was replaced by a mature squamous epithelium without cytological atypia. The grading of dysplastic lesions was in accordance with the WHO criteria; from mild dysplasia to CIS, a progressive accumulation of abnormal cells throughout the epithelium and an increase in the severity of cytological atypia were observed (31). Cases of mild and moderate dysplasia were defined as LGD, and cases of severe dysplasia and CIS were defined as HGD. Invasive carcinomas were all SCCs. Representative examples of atypia, SM, LGD, and HGD are illustrated in Fig. 1.

Immunohistochemical staining was performed on all of the lesions identified. Because of the small size of some of the lesions, there was inadequate material to study all of the cell cycle regulators in some cases. For cyclin D1, p53, and Rb detection, there were 36 cases of normal bronchial epithelium, 34 cases of atypia, 28 cases of SM, 17 cases of LGD, 30 cases of HGD, and 36 cases of SCC available for study. For cyclin E detection, there were 24 cases of normal bronchial epithelium, 22 cases of atypia, 16 cases of SM, 12 cases of LGD, 21 cases of HGD, and 24 cases of SCC available for study.

Selected blocks of bronchial lesions (along with the available corresponding...
invasive SCCs) were sectioned (4–5-μm thick), deparaffinized, and rehydrated. The antibodies, sources, dilutions, and pretreatment conditions used are shown in Table 1. Standard streptavidin-biotin-peroxidase detection techniques were used, with diaminobenzidine as the chromogen. For p53, cyclin D1, and cyclin E, cases showing nuclear staining in 10% or more of the nuclei within the lesion were scored as positive. Staining for Rb was considered negative (i.e., abnormal) when a complete absence of stain was found in the tumor cells in the face of positive staining of stromal, lymphoid, or nonneoplastic epithelial cells. To assess the proliferative rates of the tumor cells, staining of SCC was performed using the Mib-1 antibody, which detects a nuclear antigen present only in proliferating cells (32, 33). The Mib-1 antibody stain was scored as the percentage of tumor cells showing nuclear positivity.

RESULTS

Staining Patterns in Normal Bronchial Epithelium

The normal ciliated bronchial epithelium and other nonneoplastic tissues (i.e., peribronchial glands, alveolar pneumocytes, stromal tissues, and lymphoid cells) did not exhibit nuclear expression of cyclin D1, cyclin E, or p53. Occasional cytoplasmic staining for cyclin E was found in endothelial cells and vascular smooth muscle cells. Rb staining was detected in 5–10% of normal bronchial epithelial cell nuclei, generally those in the basal aspects of the epithelium. Staining of scattered cells for Rb was also found in nonneoplastic stromal and lymphoid cells.

Staining Patterns in Invasive Carcinoma

The proportion of tumor cells that stained in the positive cases varied with the antibodies used; 10–25% of the cells were positive for cyclin D1, 15–60% of the cells were positive for cyclin E, and 20–80% of the cells were positive for p53 in the cases interpreted as positive overall (i.e., those with more than 10% of cells stained). The normal immunophenotypic staining pattern of Rb was nuclear positivity in 20–80% of tumor cells; abnormal staining was defined as the absence of immunoreactivity in any of the tumor cells. Representative cases exhibiting aberrant staining for cyclin D1, cyclin E, and p53 are illustrated in Fig. 2.

As shown in Table 2, the most frequent alteration observed was p53 accumulation, which was present in 22 of 36 cases (61%). The next most common changes were overexpression of cyclin E in 13 of 24 cases (54%) and overexpression of cyclin D1 in 15 of 36 cases (42%).

| Table 1 Antibodies used, dilutions, and pretreatment conditions |
|----------------------|----------------------|----------------------|----------------------|
| Antigen | Antibody | Source | Dilution | Pretreatment |
| Cyclin D1 | Ab-3 monoclonal | Oncogene Science | 1:200 | Microwave |
| Cyclin E | HE12 monoclonal | Gift of Dr. Ed Harlow | 1:1000 | Microwave |
| p53 | 1801 monoclonal | Oncogene Science | 1:500 | Microwave |
| Rb | OP-66 monoclonal | Oncogene Science | 1:1000 | Microwave |
| Ki-67 | Mib-1 monoclonal | Immunotech | 1:50 | Microwave |

Fig. 1. Representative examples of (A) bronchial epithelium with atypia, (B) SM, (C) LGD, and (D) HGD.
Aberrant Rb staining was the most infrequent abnormality noted and was present in only 2 of 36 (6%) examined cases.

Expression of both cyclins D1 and E was studied in 24 cases. Eight of the cases were positive for both cyclin D1 and cyclin E, whereas nine cases were positive for only one of these cyclins. Thus, cyclin D1 and cyclin E expression occurred either in association or singly in 17 of 24 cases examined (71%). Only 7 of 24 cases (29%) were negative for both cyclins.

A comparison of the patterns of expression of cyclin D1, cyclin E, and p53 is shown in Fig. 3A. The most common alteration detected was p53 overexpression alone (present in 25% of cases), followed by aberrant expression of all three of these species (seen in 21% of

Table 2  Expression of cyclin E, cyclin D1, and p53, indicated as the number of cases with aberrant expression/total analyzed for each antigen

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<th>HGD</th>
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<td>Cyclin D1</td>
<td>0/36</td>
<td>15/36 (42%)</td>
<td>5/34 (15%)</td>
<td>2/28 (7%)</td>
<td>3/17 (18%)</td>
<td>14/30 (47%)</td>
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<tr>
<td>Cyclin E</td>
<td>0/24</td>
<td>13/24 (54%)</td>
<td>2/22 (9%)</td>
<td>0/16</td>
<td>0/12</td>
<td>7/21 (33%)</td>
</tr>
<tr>
<td>p53</td>
<td>0/56</td>
<td>22/56 (61%)</td>
<td>2/34 (6%)</td>
<td>0/28</td>
<td>0/17</td>
<td>16/30 (53%)</td>
</tr>
<tr>
<td>Rb</td>
<td>0/36</td>
<td>2/36 (6%)</td>
<td>0/34</td>
<td>0/28</td>
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cases). Alterations in both p53 and cyclin D1 were present in 28% of cases, alterations in one of the two were present in 51% of cases, and alterations in neither were present in 25% of cases. A total of 29% of cases studied had abnormalities in both p53 and cyclin E, 63% had alterations in one of the two, and only 8% had abnormalities of neither p53 nor cyclin E. Abnormalities in more than one cell cycle regulator were present in 55% of cases. Normal expression of all three cell cycle regulators was seen in only two cases (8%), 92% of cases displayed abnormalities in at least one of these three cell cycle regulators. Overexpression of at least one of these cyclins was observed more frequently in the presence of p53 overexpression (10 of 16 cases) than in its absence, although 6 of 16 cases showed positive staining for cyclin D1 or cyclin E in the absence of p53 staining. Thus, both cyclin D1 overexpression and cyclin E overexpression occurred in either the presence or absence of p53 abnormalities. Both of the cases exhibiting Rb abnormalities had normal cyclin D1 and p53 expression. One of them was studied for cyclin E, which showed overexpression.

Staining Patterns in Bronchial Lesions

Atypia. Bronchial atypia showed infrequent abnormalities in the expression of these cell cycle regulators. The most common alteration observed was cyclin D1 expression, which was found in 5 of 34 cases (15%); cyclin E expression was detected in 2 of 22 cases (9%), and p53 accumulation was detected in 2 of 34 cases (6%) (Table 2). Overexpression of at least one cyclin D1, cyclin E, or p53 occurred in only 4 of 22 cases (18%) analyzed for all three regulators; one cyclin D1-positive case was also positive for cyclin E; all other abnormalities occurred singly. Rb was normal in all 34 cases studied.

SM. SM also exhibited infrequent abnormalities. The only species showing abnormal expression was cyclin D1, which was aberrantly expressed in 2 of 28 cases (7%). Cyclin E (0 of 16 cases), p53 (0 of 28 cases), and Rb (0 of 28 cases) showed normal patterns of expression.

LGD. The only cell cycle regulator observed to have altered expression in LGD was cyclin D1, which was overexpressed in 3 of 17 cases examined (18%). Expression patterns of cyclin E (0 of 12 cases), p53 (0 of 17 cases), and Rb (0 of 17 cases) were normal.

HGD. In contrast to LGD, HGD commonly exhibited alterations in p53, cyclin D1, and cyclin E expression in frequencies approaching those of invasive carcinoma. The most common abnormality was p53 accumulation, which was observed in 16 of 30 cases (53%), followed by cyclin D1 overexpression in 14 of 30 cases (47%) and cyclin E overexpression in 7 of 21 cases (33%). Rb expression was normal in all 30 cases studied (see Table 2).

A comparison of the expression patterns of p53, cyclin D1, and cyclin E in HGD is shown in Fig. 3B. Seven of 11 cases positive for cyclin D1 were negative for cyclin E, whereas the few cases positive for cyclin E were fairly evenly distributed between cyclin D1-positive cases (4 of 7) and cyclin D1-negative cases (3 of 7). Thus, coexpression of both of these cyclins was not common (4 of 21 cases). Only 7 of 21 cases analyzed for both cyclins showed no overexpression of either species.

Comparing changes in cyclin D1 and p53, 23% of cases had both regulators, 53% had one or the other, and 23% had neither. Comparing cyclin E and p53 expression patterns, 19% of cases had abnormalities in both, 52% had abnormalities in one, and 29% had abnormalities in neither. Thus, no association was evident between staining for p53 and overexpression of either cyclin D1 or cyclin E in HGD. Overexpression of at least one of these species was detected immunohistochemically in 19 of 21 cases (91%). The most common alterations were p53 accumulation alone in 5 of 21 cases (24%) and aberrant cyclin D1 expression alone in 4 of 21 cases (19%). More than one abnormality was found in 41% of the cases.

Expression of Cell Cycle Regulators in Preneoplastic Lesions versus Matched Invasive Carcinomas

Atypia, SM, and LGD. The only marker in this series showing aberrant expression in atypia, SM, or LGD was cyclin D1. Of the three atypia cases that were cyclin D1 positive, the corresponding SCC was negative in two cases and positive in one case. Both cyclin D1-positive SM cases were negative in their matched SCC. Of the three cyclin D1-positive LGD cases, two were positive and one was negative in the SCC.

HGD. Seventeen available cases exhibiting foci of HGD associated with SCC were analyzed for cyclin D1 and p53. In all seven cyclin D1-positive cases of SCC, the HGD component was also positive. One case exhibited cyclin D1 expression only in the HGD; no cases were positive for cyclin D1 only in the SCC. Ten of 12 p53-positive cases of SCC (83%) were also positive in the HGD component, and 2 of 12 cases (17%) exhibited p53 staining only in the
SCC. No cases displayed p53 positivity only in the HGD lesion. Nine paired HGD-SCC cases were available for analysis of cyclin E expression. All positive cases (4 of 4) were positive in both HGD and SCC.

**Correlation between Cyclin Expression and Proliferative Rate**

It was theorized that IHC staining for cyclins D1 and E might merely reflect the proliferative rate of the tumors. If this were true, the cyclin-positive SCCs would have a higher proliferative rate than the cyclin-negative tumors. To address this question, the proliferative rate of SCCs was determined as the fraction of tumor cell nuclei staining positive by IHC for the Ki-67 antigen, which is only expressed by proliferating cells but not by cells in G0 (32). For this purpose, 31 cases studied for cyclin D1 and 22 cases studied for cyclin E were also stained with the Mib-1 antibody recognizing Ki-67. Based on Mib-1 staining, the proliferative rates were comparable between cyclin-positive and cyclin-negative cases. The proliferative rates averaged 52% in cyclin D1-positive cases (11 of 31) and 46% in cyclin D1-negative cases (20 of 31); they were 60% in cyclin E-positive cases (12 of 22) and 54% in cyclin E-negative cases (10 of 22). The number of samples examined and the methods used (IHC) do not allow formal statistical analysis, but these results strongly suggest that overexpression of cyclins D1 and E is not related solely to changes in the proliferative rate. Although all SCCs showed proliferative rates ranging from 20 to >90%, positive staining for one or both cyclins was found only in 71% of cases.

**DISCUSSION**

Deregulation of the cell cycle is an essential aspect of neoplasia. The genes p53, Rb, cyclin D1, and cyclin E are involved in regulating the transition from G1 to the S phase of the cell cycle. Alterations of cyclin D1, p53, and Rb are all described in overt NSCLCs (5–7, 11, 12). To our knowledge, only one preliminary report of cyclin E overexpression in NSCLC exists (15). Although several reports describe frequent p53 abnormalities in bronchial preneoplastic lesions (7, 28–30), the status of cyclin D1, cyclin E, and Rb in these lesions is unknown, as are their comparative patterns of expression. It appears that abnormalities in at least one cell cycle regulator are present in most lung cancers, yet the frequency of abnormalities of more than one of these species is unclear. Whether different abnormalities may develop independently or as a consequence of alterations in other cell cycle regulators is also unknown. The current study was undertaken to determine when in the sequence of preneoplastic progression altered expression of these cell cycle regulators occurs and whether cooperative (or dependent) abnormalities of the different species occur during lung carcinogenesis.

Nuclear accumulation of p53 has been described previously in bronchial dysplasia, with increasing frequency as the degree of dysplasia progresses. The percentage of cases of HGD showing p53 accumulation in this study (52%) is similar to that found by others (28) and to that which we reported previously (7). Although p53 overexpression is very frequent in HGD and invasive carcinoma, we have found it to be infrequent in earlier lesions, suggesting that p53 plays a later role in lung tumorigenesis. However, other investigators found immunohistochemical positivity for p53 in 29% of mild dysplasias and 27% of moderate dysplasias. One explanation for this discrepancy could lie in the criteria used for IHC positivity. Whereas we defined only those cases exhibiting staining of ≥10% of nuclei as positive, others may have regarded any nuclear staining as a positive result. Bennett et al. (28), in particular, do not state the minimal percentage of positive nuclear staining accepted to score a lesion as positive. In studies reporting the percentages of cells showing positivity (28, 29), the only lesions showing staining of ≥10% of cells were severe dysplasias. Alternatively, differences in the criteria for the diagnoses of LGD and HGD may have contributed to apparent differences in p53 expression in preneoplastic lesions.

*In vitro* and *in vivo* findings indicate that cyclin D1 overexpression and cyclin E overexpression contribute to transformation. Cyclin D1 overexpression induces transformation *in vitro* (34, 35). *In vivo*, cyclin D1 overexpression is found in invasive carcinomas, including SCC of the head and neck and mammary carcinoma (36–41), in addition to NSCLC (9, 10). Overexpression of cyclin D1 is also reported in preinvasive lesions in the breast, where it is found in CIS (42). In addition, targeted transgenic overexpression of the cyclin D1 gene in the breast causes hyperplasia and carcinoma (43, 44). Cyclin E protein levels are increased in invasive breast carcinomas (17, 18), and targeted transgenic overexpression of cyclin E also induces hyperplastic and neoplastic changes in the breast (19). These data support the view that the overexpression of cyclin D1 and cyclin E observed by IHC techniques in the current study contributes to transformation in squamous neoplasia in the lung.

The current study found that histologically normal bronchial epithelium does not stain immunohistochemically for cyclin D1 and that overexpression is infrequent in bronchial atypia (15%), SM (7%), and LGD (18%) and is more frequent in HGD (47%) and SCC (42%). Thus, a pattern of increasing frequency of aberrant cyclin D1 expression was found in the progression from SM to LGD to HGD, suggesting that overexpression of cyclin D1 may constitute an early step in SCC development. The percentage of cases of SCC overexpressing cyclin D1 by IHC in the current study (42%) is similar to that found by others (42–43%; Refs. 9 and 10); however, one of these studies also considered cases showing cytoplasmic staining to be positive (9), which accounted for the majority of the positive cases. The rate of nuclear overexpression they found (28%) was lower than that observed in the current study (9). The reason for this discrepancy may relate to the use of different antibodies with differing sensitivities in these studies.

The present study reveals that cyclin E is not detected by IHC in normal bronchial epithelium, SM, and LGD and is infrequently positive in atypia (9%). In contrast, overexpression occurs in HGD (47%) and in the majority of SCCs (54%), similar to the findings for p53 staining. These data indicate that overexpression of cyclin E constitutes a later event than overexpression of cyclin D1 in the pathogenesis of lung SCC. They corroborate our recent *in vitro* findings that cyclin E overexpression plays a key role in the transformation of bronchial epithelium (16).

Overexpression of cyclins D1 and E occurred with or without p53 overexpression. In HGD and SCC, where all three species were frequently expressed, no specific association between overexpression of cyclin D1 or E with p53 was observed (Fig. 3). Specifically, there were cases exhibiting alterations in these cyclins without overexpression of p53, suggesting that cyclin D1 and E overexpression occurs independently of p53. Another observation supporting this hypothesis is that cyclin D1 overexpression was occasionally observed in LGD, preceding the development of p53 abnormalities. There was also no association between overexpression of cyclin D1 and overexpression of cyclin E. These findings suggest that multiple independent mechanisms of cell cycle deregulation may be active during lung carcinogenesis. The greater percentage of invasive SCC cases showing abnormalities of all three species may reflect the accumulation of alterations that occur with the acquisition of a fully malignant phenotype.

The nearly universal finding of abnormalities in at least one of the cyclins studied or p53 confirms the importance of cell cycle dereguration.
lation in lung tumorigenesis. The two cases each of HGD and SCC that lacked abnormalities in any of these species may have had alterations of other cell cycle regulators that were not examined in this study. Interestingly, in one of the cases of SCC with abnormal expression of Rb, both cyclin D1 and p53 had normal patterns of expression; unfortunately, cyclin E could not be studied in that case due to limited specimen availability.

In most instances, the invasive carcinomas had the same pattern of abnormalities in the cell cycle regulators as the corresponding HGD lesions. This high level of concordance suggests that the preneoplastic lesions examined were likely to relate directly to the corresponding invasive carcinoma rather than representing genetically distinct foci of preneoplastic change and reflect the sequence of cumulative genetic events responsible for the development of an invasive cancer. Examination of the specific mutations (e.g., in p53) at the genotypic level in the HGDs and corresponding SCCs would be useful to explore this hypothesis.

Alterations of cyclin D1 and p53 were found in a small percentage of bronchial atypia cases. The importance of this finding is unclear. Ambiguity exists in the pathology literature over the term “atypia”; in some cases, it is used to designate dysplasia (22), and in other cases, it is used to designate epithelium with changes that fall short of frank dysplasia and may be reactive in nature (7). In this study, atypia was used to designate a lesion with abnormal nuclear features but preserved cilia, a lesion that would not be classically included in the sequence of preneoplastic changes in the respiratory epithelium. Whereas a subset of these lesions was probably reactive in nature, the possibility that at least some of these lesions may progress to SM or LGD cannot be excluded. The occasional finding of aberrant expression of cyclin D1, cyclin E, and p53 would support the latter possibility. In fact, lesions designated as atypia in this study overlap at least partially with the lesions described by Auerbach et al. (21) as “lesions with preservation of ciliated epithelium,” these authors found an increased incidence of this type of lesion with increasing exposure to cigarette smoke (21), further supporting the potential preneoplastic nature of bronchial atypia.

Rb alterations were found in this study only in overt SCCs in 6% of cases. This percentage is somewhat lower than the 15% rate observed by others in NSCLC (11, 12) but reemphasizes that Rb abnormalities in invasive carcinomas are likely to relate directly to the corresponding HGD lesions examined. In the invasive carcinomas, increased incidence of this type of lesion with increasing exposure to cigarette smoke (21), further supporting the potential preneoplastic nature of bronchial atypia.

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