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Genetics

A model for tumor suppression using H-1 parvovirus

(cancer genetics/tumor suppressor genes/p53/oncogenes)

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ABSTRACT A model system is proposed to investigate, at the molecular level, the pathways of tumor suppression. As a tool for the selection of cells with a suppressed phenotype, we used the H-1 parvovirus that preferentially kills various neoplastic cells. From the human K562 leukemia cells, we isolated a clone, KS, that is resistant to the cytopathic effect of the H-1 virus and displays a suppressed malignant phenotype. The suppressed malignancy and the cellular resistance to H-1 killing appear to depend on the activity of wild-type p53. Whereas the KS cells express wild-type p53, the protein is undetectable in the parental K562 cells. Experiments with p53 mutants suggest that wild-type p53, in its functionally intact state, contributes to the resistance against the cytopathic effect of H-1 parvovirus.

Chemical mutagens, viruses, and cellular oncogenes have been widely investigated in relation to their role in malignant transformation (1). Far fewer systems have been made available to study tumor suppression (2). The evidence for the existence of tumor suppressor genes comes from experimental studies, statistical analysis of mutations in tumors, and cloning of genes located in chromosomal regions deleted in particular cancers (3–14). This experimental evidence came first from the analysis of somatic cell hybrids (3). It has been demonstrated that fusion between malignant and nonmalignant mouse cells resulted in hybrids unable to form tumors. This led to the hypothesis that the malignant phenotype could be suppressed by the introduction of normal genetic information. This hypothesis was further strengthened by single chromosome transfer (7). More recently, gene transfer definitely confirmed that the malignant phenotype could be suppressed experimentally (9, 10, 12, 13). The purpose of our work was to establish an experimental model to study tumor suppression, consisting of a parental human tumor cell line and a directly derived daughter cell line exhibiting suppressed malignancy. The availability of such a system would enable investigations at the molecular level by techniques such as subtraction hybridization. To obtain such a system, we have used the H-1 parvovirus, a small single-stranded DNA virus that has the property of preferentially killing a variety of tumor cells (15–19). Hence our strategy was to kill the malignant cell population with H-1 virus and to rescue the resistant cells. Those cells resistant to the cytopathic effect of H-1 could have undergone the changes leading to a suppressed malignancy. In the present study, we applied this strategy to select cells with a suppressed malignant phenotype derived from the K562 leukemia cell line. The role of the p53 gene in this model has been investigated.

MATERIALS AND METHODS

Selection and Characterization of KS Cells. Single clones derived from a K562 cell culture have been infected with H-1 parvovirus using standard procedures (19). The cytopathic effect of the virus caused massive cell death, sparing a single resistant clone (KS). For the measurement of cell survival, 5 $\times 10^5$ cells were inoculated with H-1 virus at a multiplicity of infection (MOI) of 100 plaque-forming units (PFUs) per cell for 1 h at 37°C and further incubated in culture medium. Survival is defined as the relative number of living cells in H-1 virus-infected versus mock-treated cultures, as measured 1 week after infection (20). For the measurements of virus uptake, 2×10^6 cells were incubated in the presence of ^{32}P-labeled H-1 virus (MOI = 0.5 PFU per cell; 0.1 cpm per PFU) for 1 h at 37°C. Total cell-associated radioactivity was taken as a measurement of virus binding (20). Bound virus that has not been internalized can be removed from the cell surface with EDTA, a fact that permits the use of EDTA-elution-resistant radioactivity for the measurement of virus penetration (20).

To detect viral DNA in KS cells, PCR analysis was performed using the following primers: 1, 5′-GACTGCCTGTAATGTTCAA-3′; 2, 5′-TGCTCACTAGATGGCGCTCG-3′, 3, 5′-CAGCCAGAGTCACTGCTAAG-3′. The combination of primers 1 and 2 gives a PCR product of 276 bp and the combination of primers 1 and 3 gives a PCR product of 230 bp. For Southern blot analysis and *in situ* hybridization, a virus-specific probe corresponding to the NS domain was used. Titers of H-1 virus released from KS cells were determined by an infectious center assay. Briefly, indicator monolayer cultures of NBE cells (10^5 cells per ml) were overlaid with a mixture of agar and medium containing various numbers of KS cells, and plaque formation was visualized after 6 days.

Surface antigen phenotyping (21, 22) and DNA fingerprints (23, 24) were performed as described. For the cell cycle analysis, DNA content was measured by fluorocytometry (25).

To measure the colony-forming efficiency of K562 and KS cells, different number of cells were seeded in 0.38% agar. Colonies were scored for size and number after 3 weeks (means and SD from three experiments).

To measure the *in vivo* tumorigenicity of K562 and KS cells, *scid/scid* mice (7–8 weeks old) were injected subcutaneously in the flank fat pads with cell suspensions. The tumorigenicity is expressed as the number of injected animals that developed a tumor within 2 months. The delay is the

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Abbreviations: MOI, multiplicity of infection; PFU, plaque-forming units; REF, rat embryo fibroblast.
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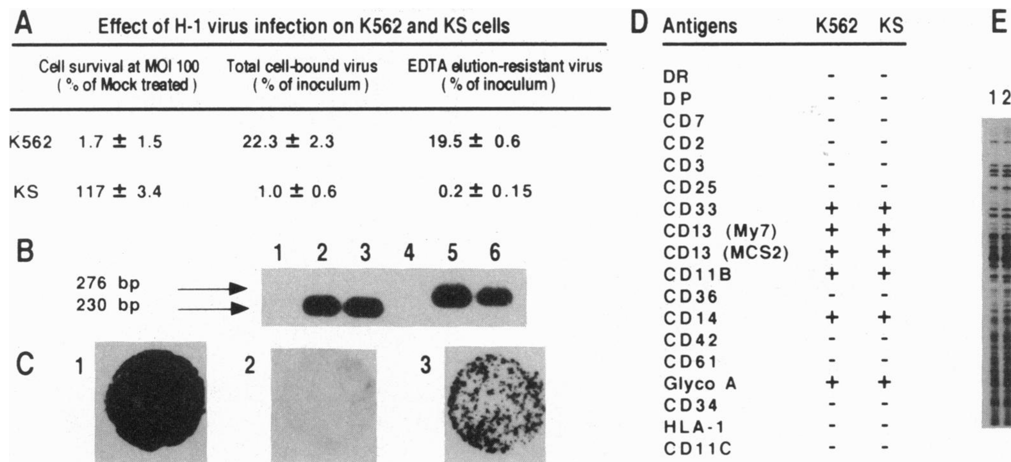

FIG. 1. Characterization of the KS cells. (*A*) A striking cytopathic effect was observed in the K562 culture (clone WA) that progressively degenerated in the weeks after infection, whereas the growth of KS cells was unaltered. (*B*) To detect the presence of viral DNA in KS cells, a PCR from genomic DNA was performed, using two sets of primers. No signal was detectable in the genomic DNA from K562 cells (lanes 1 and 4). Fragments of 230 or 276 bp are generated from the DNA of KS cells (lanes 2 and 5) and control viral DNA (lanes 3 and 6). (*C*) The release of H-1 virus by KS cells was assessed by incubating NBE cells with conditioned medium from a KS culture, followed by *in situ* hybridization with a probe corresponding to the NS region of parvoviral DNA. Cultures: 1, positive control of NBE cells infected directly with H-1 virus at a MOI of 1 PFU per cell; 2, NBE cells, infected with conditioned medium from K562 cells; 3, NBE cells infected with conditioned medium from KS cells. (*D*) The stage of differentiation was assessed by determining whether (+) or not (−) cells reacted with antibodies directed against indicated specific surface proteins. (*E*) The DNA fingerprints of K562 (lane 1) and KS (lane 2) cells were obtained by *Hin*fI digestion of genomic DNA and hybridization to the multilocus C probe.

average time after which at least half of the tumors appeared in a series.

Protein Analysis by Two-Dimensional Gel Electrophoresis and Immunodetection. K562 and KS cells were labeled *in vivo* with [^{35}S]methionine (26) and proteins were separated as described (27). Alternatively, proteins were silver stained. Immunoprecipitation followed by *in vitro* phosphorylation was performed using the anti-bcr and anti-abl antibodies as described (26). Western blot analysis was performed with the monoclonal antibodies against p210 (Oncogene Science), anti-pim-1 (22), anti-myc (Oncogene Science), and anti-p53 (Oncogene Science).

Transfection of Rat Embryo Fibroblasts (REFs) with p53 Mutants. Cells and plasmids used have been described (13, 28).

RESULTS AND DISCUSSION

KS Cells Possess a Suppressed Phenotype. We inoculated a culture of human K562 leukemia cells, derived from a single clone by limiting dilution, with H-1 virus. This treatment caused the progressive degeneration of the culture, with a reduction of the number of living cells to ≈1% of mock-treated cells after 1 week (Fig. 1*A*). Maintenance of this culture led to further degeneration of the K562 cells, sparing a single clone that was recovered 10 weeks after infection. This clone (named hereafter KS) was expanded to a continuously growing cell line. In contrast to the parental K562 cells, which were very sensitive to the viral cytopathic effect, the KS cells were stably resistant to virus infection. This resistance could be due to a down-modulation of the surface receptor or reside in a pathway located more downstream. Radiolabeled H-1 virus binds poorly to the KS cells. However, using PCR analysis, we detected viral DNA in KS cells, 1 year after infection (Fig. 1*B*). Sequence analysis of those PCR products derived from KS-cell DNA indicates a 100% identity with the viral sequence. This was confirmed by Southern blot analysis (data not shown). Moreover, the KS cells release infectious H-1 virus. Indeed, the cell-free conditioned medium from a KS culture could be used to infect NBE indicator cells, as shown by *in situ* hybridization with a H-1-virus-DNA-specific probe (Fig. 1*C*). An infectious center assay indicates that each individual KS cell produces infectious H-1 parvovirus, and the cell-free conditioned medium from KS cells, causing the death of NBE cells, was also able to induce a rapid degeneration of K562 cells (data not shown). These phenotypic changes in KS cells could have been related to a shift in differentiation. Yet, screening of 18 surface antigens, indicative of the stage of differentiation (21, 22), showed KS cells to be indistinguishable from K562 cells (Fig. 1*D*). Since KS cells produce cytopathic H-1 virus, the resistance to viral-induced killing developed by KS cells was not due to downregulation of surface receptors. To exclude any contamination of the parental K562 cell population with other cells, DNA fingerprints were performed with a multilocus probe (23). Identical patterns were observed for K562 and KS cells (Fig. 1*E*). The DNA fingerprints obtained with the pH30 monolocus probe (24) also confirmed that both cell lines are of the same origin (data not shown). The karyotypes of K562 and KS cells do not display any significant difference (data not shown).

Table 1 summarizes the growth properties of K562 and KS

Table 1. Growth properties of K562 and KS cells

	RPMI/10% FCS		Soft agar		*scid/scid* mice			
					10^6 cells		10^7 cells	
Cell	Doubling time, h	$G_{0/1}/S/G_2+M$ phase, %	CFE, %	Average colony diameter, μm	Tumorigenicity	Delay, days	Tumorigenicity	Delay, days
K562	19	44/37/19	23 ± 1.8	300 ± 50	3/5	15	5/5	15
KS	19	51/32/17	2.6 ± 0.1	76 ± 29	1/5	56	6/10	30

CFE, colony-forming efficiency; FCS, fetal calf serum. Tumorigenicity is expressed as the number of animals that developed a tumor within 2 months. Delay is the average time after which at least half of the tumors appeared in a series.

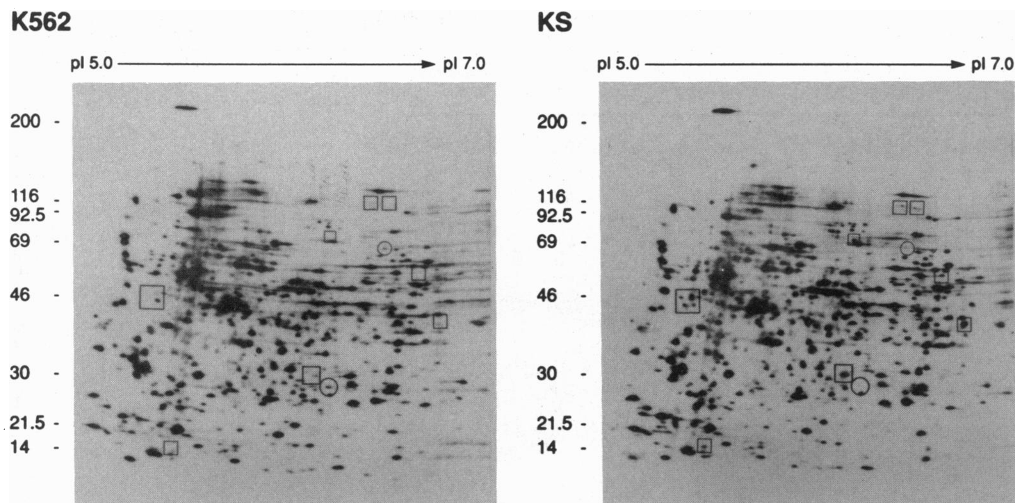

FIG. 2. Two-dimensional gel electrophoresis of proteins expressed by K562 and KS cells. K562 and KS cells were labeled *in vivo* with [^{35}S]methionine and proteins were separated by two-dimensional gel electrophoresis. Squares indicate proteins that are present in KS cells but cannot be detected in K562 cells. Circles indicate proteins detected in K562 but not in KS cells. Only those proteins that were consistently different in three experiments and could be detected by silver staining are considered. KS-specific proteins with molecular masses of about 105, 70, 50–55, 40, and 30 kDa are found toward the more basic isoelectric point, while one protein of 14 kDa and three proteins of 45 kDa are detected at the more acidic isoelectric point. The two K562-specific proteins migrate toward the more basic isoelectric point and have a molecular mass of 68 and 28 kDa, respectively.

cells. Under standard culture conditions [RPMI medium supplemented with 10% (vol/vol) fetal calf serum], both cell lines have a comparable growth pattern with a population doubling time of 19 h and similar proportions of cells in $G_{0/1}$, S, and G_2 plus M phases. Cell cycle analysis by immunohistochemistry, using the proliferating cell nuclear antigen as a marker, confirmed the above results (data not shown). In contrast, there is a striking difference between K562 and KS cells with regard to their transformed and tumorigenic properties. In soft agar, the colony-forming efficiency of KS cells is 10-fold lower than that of K562 cells, while the average diameter of colonies is 4 times smaller. Also, *in vivo* inoculation of cells in *scid/scid* mice indicates a significant difference (Mann–Whitney test, $P = 0.05$) in behavior of the two cell lines, with both a decreased number of tumors and a longer delay in the appearance of the residual tumors in mice injected with KS cells. These data indicate that the KS cells possess a suppressed phenotype.

KS Cells Reexpress Wild-Type p53. To investigate this difference in tumorigenicity at the molecular level, we first compared the overall expression of proteins by two-dimensional gel electrophoresis. Although the overall pattern of expression is similar in the two cell lines, there are subtle but significant differences in the production of particular proteins. As shown in Fig. 2, KS cells express 11 proteins that are not detected in K562 cells, and two spots present in K562 cells are missing in KS cells. We further analyzed whether some of the specific molecular changes occurring in leukemias are still present in KS cells. The t(9;22) translocation involving the *bcr* and *abl* genes has been implicated as a major defect leading to the malignant phenotype of particular leukemic cells. Since this translocation is present in the K562 line, we analyzed the p210 bcr–abl fusion protein (29, 30) in K562 and KS cells. As illustrated in Fig. 3A, a similar extent of *in vitro* phosphorylation was observed for p210 immunoprecipitated from either cell line by anti-bcr or anti-abl antibodies. Western blot analysis with anti-abl antibodies also indicated that K562 and KS cells express the same level of p210 (Fig. 3B). Two other oncogenes that are activated in leukemias, *pim-1* and c-*myc* (22), are also expressed in similar amounts by K562 and KS cells (Fig. 3 C and D). These data indicate that during suppression of the malignant phenotype, some of the proteins that have been implicated in the process of malignant transformation remain active. This was previously described with the p21 c-Ha-*ras* oncogene that continued to be expressed during suppression of tumorigenicity (32).

Recent evidence indicates that the wild-type p53 is a tumor suppressor gene (12, 13, 33) and that mutations in this gene are common in human cancer (34, 35). We therefore asked whether the suppressed malignant phenotype of KS cells could be correlated with changes in p53 expression. K562 cells do not express detectable levels of p53 (36). This was also confirmed by us (Fig. 3E). In contrast, p53 protein can be readily detected in KS cells by Western blot analysis using the anti-p53 antibodies PAb240 (31) (Fig. 3E) or PAb421 (37) (data not shown). To determine the nature of the expressed p53, we sequenced the entire p53 coding region in KS cells. Two overlapping segments of cDNA were obtained by re-

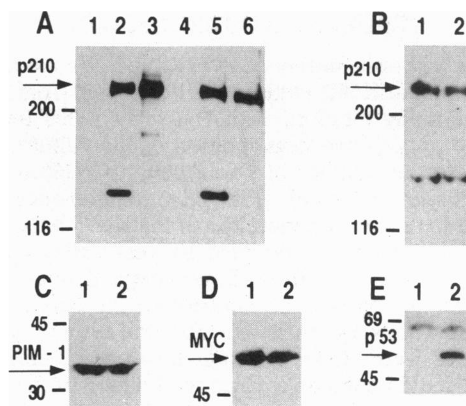

FIG. 3. Expression of the protein products from oncogenes and the p53 tumor suppressor gene. (*A*) *In vitro* phosphorylation assays were performed as described (26), using K562 (lanes 1–3) and KS (lanes 4–6) cell extracts immunoprecipitated with preimmune serum (lanes 1 and 4), anti-bcr antibodies (lanes 2 and 5), and anti-abl antibodies (lanes 3 and 6). (*B–E*) Western blot analysis of p210 (*B*), pim-1 (*C*), myc (*D*), and p53 (*E*) expression in K562 (lanes 1) and KS (lanes 2) cells. Monoclonal antibodies, respectively, directed against p210 (Ab3; Oncogene Science), pim-1 (22), myc (Ab1; Oncogene Science), and p53 (Ab3, PAb240; Oncogene Science) (31) were used.

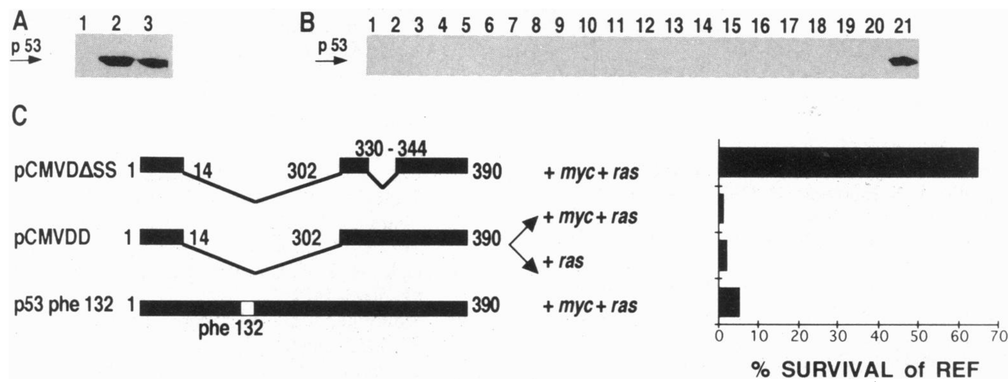

FIG. 4. Resistance to H-1 parvovirus and expression of p53. (*A*) Selection of additional clones, similar to KS cells, was performed by using the same approach as described above. From K562 cells (clone W7), two clones, KS2 and KS3, were derived, based upon their resistance to the cytopathic effect of H-1 virus. Western blot analysis on protein extracts with anti-p53 antibodies shows no expression of p53 in the K562 cells (lane 1) but p53 is easily detectable in KS2 (lane 2) and KS3 (lane 3) cells. (*B*) No p53 production could be induced in K562 cells by direct stimulation with H-1 parvovirus. Western blot analysis of protein extracts with anti-p53 at various time intervals after infection (0, 2 h, 4 h, 8 h, and 20 h). Stimulation of K562 cells in mock conditions (lanes 1–5), with H-1 at a MOI of 100 PFU per cell of full virions (lanes 6–10) or an equivalent amount of empty capsids from H-1 virus (lanes 11–15) or 3×10^{-4} PFU per cell of full virions corresponding to the residual infectivity of the empty capsids (lanes 16–20). Positive control of protein extracts from KS cells expressing the p53 (lane 21). (*C*) The cytopathic effect of H-1 virus on REFs transfected with various constructs of p53 (28) was assessed by measuring the percent of survival after H-1 infection compared to a mock-treated culture. The REFs transfected with *myc* plus *ras* plus pCMVΔSS, containing p53 sequences that do not interfere with endogenous p53, are resistant to H-1 virus. The REFs transfected with pCMVDD, which abolishes the activity of the endogenous p53 in a dominant negative fashion, are highly sensitive to H-1 virus. This is true both for cells transfected with pCMVDD plus *myc* plus *ras* and for cells transfected with pCMVDD plus *ras* only. REFs transfected with *myc* plus *ras* plus the Phe-132 mutant p53 are also sensitive to the cytopathic effect of H-1 virus.

verse transcription of KS mRNA followed by PCR. Direct sequencing of the PCR products did not reveal any mutation (data not shown), indicating that the expressed p53 is of the wild-type form that has been functionally implicated in tumor suppression.

To confirm the above results by using the same approach, we isolated, from an independent K562 culture, two clones (named hereafter KS2 and KS3) that exhibit an identical pattern of resistance to H-1-induced killing and express p53 protein (Fig. 4*A*). Clones KS2 and KS3 have a strongly suppressed malignant phenotype with no colonies formed in soft agar, as compared to the original K562 cells, and a suppression of tumorigenicity equivalent to the KS cells (data not shown). The frequency at which clones with the KS phenotype are selected was determined by limited dilution and is ≈1 in 5×10^5 cells. One possible switch leading to an expression of the p53 protein could be the direct stimulation with H-1 virus or triggering of the receptor with purified H-1 empty capsids. However, Fig. 4*B* illustrates that expression of p53 is not directly inducible by H-1 parvovirus stimulation.

p53 Mediates the Cellular Resistance to the Cytopathic Effect of H-1 Parvovirus. To investigate whether the resistance to the cytopathic effect of H-1 parvovirus is mediated by the p53 protein, we used a different cellular system. This system consisted of low-passage REFs that had been stably transformed through transfection with various combinations of *ras*, mutant or truncated p53 genes, and typically also deregulated *myc* (28). Of special interest were cells expressing C-terminal portions of p53. The C-terminal region of p53 is involved in oligomerization and DNA binding (28, 38–41). Construct pCMVΔSS contains the most C-terminal portion of p53 with an additional deletion spanning aa 330–344 (Fig. 4*C*). The miniprotein encoded by this construct will not bind the endogenous p53 produced by REFs and will not abrogate the sequence-specific DNA binding. For these reasons, it was used as a normal control. On the other hand, construct pCMVDD (Fig. 4*C*) encodes a p53-derived miniprotein that contains the most C-terminal 89 residues of mouse p53 (28). This protein can oligomerize with wild-type p53, inhibit its DNA binding activity, and presumably render it biologically nonfunctional through a dominant negative mechanism (28). Fig. 4*C* illustrates the survival of REF cells carrying each of these constructs after infection with H-1 parvovirus. Cells transfected with pCMVDD are highly sensitive to H-1 virus. Moreover, a high sensitivity to H-1-induced killing is obtained also in REFs transfected with a p53 mutated at position 132, which can also interfere with the function of endogenous rat p53 (28). These data suggest a close relationship between wild-type p53 function and resistance to H-1-induced killing. This relationship is not secondary to a modulation or down-regulation of the H-1 receptor by p53. Binding experiments, using radiolabeled H-1 virus, indicate that there is no correlation between the abundance of H-1 receptor and the nature of the p53 expressed (the percent of bound and internalized H-1 virus was 31% for REFs expressing pCMVΔSS and 29% for those expressing the p53 mutated in position 132).

In conclusion, the present study suggests that while some viruses are known to be oncogenic, the H-1 virus in contrast can be used as a valuable tool for the selection of a suppressed malignant phenotype. Moreover, at least in some experimental systems, the cellular resistance to H-1 parvovirus killing may be dependent upon a functionally intact p53 protein. A more detailed molecular analysis of the model system presented in this work could lead to the isolation of genes involved in the suppression pathway.

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