Transcriptional silencing of the Dickkopfs-3 (Dkk-3) gene by CpG hypermethylation in acute lymphoblastic leukaemia

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Dkk-3 is a newly characterised mortalisation-related gene and an antagonist of the Wnt oncogenic signalling pathway whose expression is decreased in a variety of cancer cell lines, suggesting that the Dkk-3 gene, located at chromosome 11p15.1, functions as a tumour suppressor gene. Although 11p15 is a ‘hot spot’ for methylation in acute lymphoblastic leukaemia (ALL), the role of Dkk-3 abnormalities has never been evaluated in this disease. We analysed CpG island methylation of the Dkk-3 promoter in six ALL cell lines and 183 ALL patients. We observed Dkk-3 hypermethylation in all cell lines and in cells from 33% (60/183) of ALL patients. Moreover, Dkk-3 methylation was associated with decreased Dkk-3 mRNA expression and this expression was restored after exposure to the demethylating agent 5-AzaC. Clinical features did not differ between hypermethylated and unmethylated patients. Estimated disease-free survival (DFS) and overall survival at 10 and 11 years, respectively, were 49.8 and 45.6% for normal patients and 10.5 and 15.1% for hypermethylated patients (P = 0.001 and 0.09). Multivariate analysis demonstrated that Dkk-3 methylation was an independent prognostic factor predicting DFS (P = 0.0009). Our data suggest that Dkk-3 methylation occurs at an early stage in ALL pathogenesis and probably influences the clinical behaviour of the disease.

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DNA methylation is an essential mechanism for the regulation of gene expression in mammalian cells (Bird, 2002). Methylation occurs at cytosine residues within CpG dinucleotides and many genes are enriched with these dinucleotides in their promoters. These regions are known as CpG islands and are generally nonmethylated, a condition that allows genes to be transcriptionally competent. Methylation of CpG islands within gene promoters leads to transcriptional silencing through recruitment of methyl-CpG binding protein and histone deacetylases (Jones and Baylin, 2002). Hence, identification of the methylation patterns of CpG islands in mammalian cells is important for understanding normal and pathological gene expression. Several reports have shown that abnormal hypermethylation of CpG islands may contribute significantly to the pathogenesis of human leukemias. Hypermethylation of promoters associated with genes involved in the control of the principal late-G1 cell-cycle checkpoint, the apoptotic programme and the cell-cell adhesion is detected with increased frequency in acute lymphoblastic leukaemia (ALL) (Roman-Gomez et al, 2003). This mechanism provides an alternative route to gene mutation of cancer-related genes. Although genomewide methylation changes in CpG islands are early and frequent events in ALL, it is also evident that some of these changes occur preferentially in specific chromosome regions. In this context, 11p15 region seems to represent a ‘hot spot’ for methylation in ALL. In fact, epigenetic inactivation of Calcitonin (11p15.1 – 15.2), p57 (11p15.5) and Myf-3 (11p15.4) genes are frequently observed in both childhood and adult ALL (Roman-Gomez et al, 2001; Garcia-Manero et al, 2002; Li et al, 2002). Interestingly, Calcitonin gene is methylated in a large proportion of ALL patients with dismal prognosis. However, this gene is generally considered not to be causally related to the malignant process suggesting that its methylation is a surrogate for the methylation of another potential tumour suppressor gene located in its vicinity.

The human Dickkopfs-3 (Dkk-3) gene, located on chromosome 11p15.1 is a recently found mortalisation-related gene expression of which is largely attenuated in many immortalised and tumour-derived cell lines (Tsujii et al, 2000). Furthermore, by transfection experiments, it has been determined that Dkk-3 possesses an antiproliferative activity against tumour cells, suggesting that Dkk-3 may function as a tumour suppressor (Tsujii et al, 2001).

Recent studies have shown that some DkkS play a role in a wide range of normal and pathological developmental processes, including cancer, and that their effects seem to be mediated by their ability to antagonise Wnt signalling (Taipale and Beachy, 2001). Wnt signalling pathway has profound effects in normal haematopoiesis inducing bone marrow cells to develop into a variety of different lymphoid cell types and also augmenting

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repopulating capacity and primitive hematopoietic development of human blood stem cell in vivo (Brandon et al, 2000). In addition, expression of Wnt-16 following a chromosomal translocation has been reported in preB-cell ALL (McWhirter et al, 1999) and Wnt5a hemizygous mice develop myeloid leukemias and B-cell lymphomas that are clonal in origin suggesting a role for Wnts in leukaemogenesis. However, little is known about the potential role of Wnt antagonists in lymphoid neoplasia.

In this paper, we demonstrate that the putative tumour suppressor Dkk-3 gene is silenced by methylation in ALL and that this epigenetic event confers poor prognosis in this group of patients.

MATERIALS AND METHODS

Cell lines and samples

Four human precursor-B ALL (BV173, TOM-1, MY and NALM-20) and two T-cell leukaemia (MOLT-3 and MOLT-4) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in the appropriate medium until harvested for extraction of DNA and RNA using standard procedures. Bone marrow samples obtained by aspiration were collected after an acquisition of informed consent from 183 consecutive patients (106 male; 77 female) who were diagnosed with de novo ALL between January 1989 and December 2002. Mononuclear cells were isolated from samples at diagnosis by a Ficoll–Paque gradient as per the manufacturer’s instructions (Amer sham Pharmacia Biotech AB, Uppsala, Sweden). In all the cases, the diagnostic bone marrow sample contained blast cells in the ratio of at least 70%.

The median age at diagnosis in the study population as a whole was 14.5 years (range, 0.5–82 years). Of these patients, 92 were children (median age, 5 years; range, 0.5–14) and 91 presented adult ALL (median age, 35 years; range, 15–82). Diagnosis was established according to standard morphologic, cytochemical and cytogenetic criteria. Patients were studied at the time of initial diagnosis; were risk-stratified according to the therapeutic protocol used, which was always based on recognised prognostic features (including cytogenetics); and were entered in ALL protocols of the ‘Programa para el estudio y tratamiento de las hemopatías malignas’ (PETHEMA) Spanish study group. For statistical analyses, children were also grouped according to the National Cancer Institute (NCI) risk-classification criteria (Smith et al, 1996). The specific PETHEMA ALL treatment protocols in which these patients entered included ALL-89 (between 1989 and 1994; n = 53) and ALL-93 (between 1994 and 2002; n = 130). The design and results of these studies have been previously reported (Ortega, 1998; Ribera et al, 1998; Ortega et al, 2001; Ribera et al, 2002). In all, 64 patients relapsed. Overall, 29 patients received stem cell transplantation (10 autologous, 19 allogeneic) in the first (n = 12) or second complete remission (CR) (n = 17). There are 91 patients currently alive. Clinical characteristics of the patients are listed in Table 1. Some of these patients (n = 105) were typed previously for methylation of the Calculcinin gene (Roman-Gomez et al, 2001).

Quantitative real-time PCR (qrt-PCR) for Dkk-3 expression

Total RNA was extracted from mononuclear marrow cells with Ultraspec (Biotecx, Houston, TX, USA) following the manufacturer’s instructions. Reverse transcription was performed on 1 µg total RNA, after heating at 70°C for 5 min, with random hexamers as reaction primer. The reaction was carried out at 42°C for 45 min in the presence of 12 U Avian Myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Germany). qrt-PCR was performed in a rapid fluorescent thermal cycler with three-colour fluorescence monitoring capability (LightCycler, Roche), using 1 µl of cDNA in 20 µl reaction volume with 0.4 µmol l⁻¹ each primer, and 2 µl of 10× LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The final Mg²⁺ concentration in the reaction mixture was adjusted to 3.5 mmol l⁻¹. Primer set was specific for the Dkk-3 gene (Homo sapiens Dkk-3 mRNA, GeneBank: AB033421; sense, 5′-CTGTGTGCTGGGGTACCTG-3′; antisense, 5′-GCTTACGCCTCCAGGTAGT-3′). The following programme conditions were applied for qrt-PCR running: denaturation programme, consisting of one cycle at 95°C for 8 min; amplification programme, consisting of 45 cycles at 95°C for 5 s, 60°C for 10 s and 72°C for 15 s; melting programme, one cycle at 95°C for 0 s, 40°C for 60 s and 90°C for 0 s; and cooling programme, one cycle at 40°C for 60 s. The temperature transition rate was 20°C s⁻¹, except in the melting programme, which was 0.4°C s⁻¹ between 40 and 90°C.

Amplification of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcript was performed to assess RNA integrity and as a reference gene (forward, 5′-TGAAGTTCGAGTCACCAG-\(\text{\textasciicircum}T\)-GATTTCGT-3′; reverse, 5′-CATGTGCGGCACTAGGGTCAAC-3′). It was amplified in the same run and following the same procedure described above for Dkk-3. In order to reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes vs their controls in relation to the reference gene was used. Calculations were automatically performed by LightCycler software (RealQuant, version 1.0, Roche). The normalised ratio was obtained from the next equation and expressed as percentage of the control:

\[
\text{Normalised ratio } (N_{\text{Dkk-3}}) = \left(\frac{E_{\text{target}}}{E_{\text{ref}}}\right)^{\Delta C_p \text{target}/\text{control sample}} \div \left(\frac{E_{\text{ref}}}{E_{\text{ref}}}\right)^{\Delta C_p \text{ref}/\text{control sample}}
\]

Efficiencies (E) of each gene were calculated from the slopes of crossover points (Cp) vs cDNA concentration plot, according to the formula \(E = 10^{(-1/\text{slope})}\). \(\Delta C_p\) corresponded to the difference between control Cp and sample Cp, either for the target or for the reference genes. The selected control was the bone marrow specimen from a healthy donor. It was considered as 100% expression. Occasionally, equal amounts of PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualised under UV light.

Methylation-specific PCR (MSP) of Dkk-3 promoter

Analysis of the Dkk-3 gene (GeneBank: AB045205, Homo sapiens Dkk-3 gene) has revealed that Dkk-3 promoter was CpG-rich, showing >60% C+G content and an observed-over-expected CpG frequency of >0.6, satisfying the criteria for a CpG island. aberrant promoter methylation of Dkk-3 gene was determined by method of MSP as previously reported (Her ma n et al, 1996). MSP distinguishes unmethylated alleles of a given gene based on DNA sequence alterations after bisulphite treatment of DNA, which converts unmethylated but not methylated cytosines to uracils. Subsequent PCR using primers specific to sequences corresponding to either methylated or unmethylated DNA sequences was then performed. Primer sequences of Dkk-3 for the unmethylated reaction were forward (5′-TTTGGTTTTTGTGGTTTGGG-3′) and reverse (5′-CACAACGACTACATCTCCACT-3′), which amplify a 179-bp product. Primer sequences for the methylated reaction were forward (5′-GCGTTTGTGTTTGGTTGGG-3′) and reverse (5′-GCAACGCCGCACTACATCTCCACT-3′), which amplify a 175-bp product. Briefly, 1 µg of genomic DNA were denatured by treatment with NaOH and modified by sodium bisulphite. DNA samples were purified using Wizard DNA purification resin (Promega Corp., Madison, WI, USA), treated with NaOH, precipitated with ethanol, and resuspended in 20 µl of water. A volume of 2 µl of modified DNAs were PCR amplified in a
RESULTS

**Dkk-3 expression is downregulated in ALL samples and T/B-precursor ALL cell lines**

To determine the cutoff point for altered Dkk-3 expression in ALL samples, the N\textsubscript{DDK-3} value for Dkk-3 was determined in 20 bone marrow samples from healthy donors. N\textsubscript{DDK-3} values fell between 107 and 86% (mean: 96.5±5.6%). A N\textsubscript{DDK-3} value below 68.5% (determined as the mean ± 5 s.d.) was chosen to define under-expression of Dkk-3 in ALL RNA samples. No Dkk-3 expression was observed in T- or B-precursor ALL cell lines (Figure 1B). In addition, we found a strong reduction of Dkk-3 mRNA in 41% of diagnostically ALL samples (N\textsubscript{DDK-3} mean: 23%; range, 47–0%). No overexpression of Dkk-3 (N\textsubscript{DDK-3} above 107%) was observed in ALL primary tumours.

**Methylation of Dkk-3 promoter is associated with reduced mRNA expression**

To investigate a mechanism for the reduced expression of the Dkk-3 gene, we analysed Dkk-3 promoter, which is known to be methylated in cancer cell lines lacking Dkk-3 expression (Kobayashi et al., 2002). Promoter of the Dkk-3 gene has a typical CpG island showing >60% C+G content and an observed-expected CpG frequency of >0.6. By MSP, CpG island of the Dkk-3 promoter was revealed to be highly methylated in all ALL cell lines (Figure 1A) and in 60 of 183 (33%) samples from patients with ALL (Figure 1C), which was clearly in contrast to no Dkk-3 methylation in normal cells.

All 60 methylated ALLs (100%) showed decreased Dkk-3 expression; in contrast, decreased Dkk-3 expression was detected in only 10 of 123 ALLs with unmethylated pattern (8%). This result indicated that CpG methylation within Dkk-3 promoter strongly correlated with decreased constitutive expression of Dkk-3 in ALL cells (P<0.0001).

Exposure to different concentration of the demethylating agent 5-Aza-2’-deoxycytidine restored, in both cell lysate and culture supernatant, the expression of Dkk-3 protein in the MY ALL cell line indicating that hypermethylation is a major mechanism by which Dkk-3 expression is silenced in ALL cells (Figure 1D).

Dkk-3 methylation, clinical presentation and outcome: Dkk-3 methylation was detected at diagnosis in 33% (60 out of 183) of patients with adult or childhood ALL of all the FAB subtypes. As shown in Table 1, aberrant Dkk-3 methylation was similarly observed in adults (30 out of 91, 33%) and children (30 out of 92, 32%). Clinical and laboratory features did not differ significantly between methylated and normal patients. Poor-risk cytogenetics or molecular events, risk groups according to both NCI or PETHEMA classifications, good risk features (hyperdiploidy and TEL-AML1 fusion), type of PETHEMA protocol administered and number of patients who received stem cell transplantation were similarly distributed between the two groups defined by Dkk-3 methylation. Separate analysis of adult and childhood ALL patients gave the same results as the global series. The presence of methylation in the Calcitonin gene was determined previously for 105 of these cases (Roman-Gomez et al., 2001). There was a significant concordance between Dkk-3 methylation and the presence of Calcitonin methylation; methylation was present in 30 out of 42 (71.4%) Calcitonin-methylated tumours vs 13 out of 63 (20.6%) Calcitonin nonmethylated tumours (P=0.0001).

Table 1 details the relapse history, CR rates and mortality for patients who exhibited Dkk-3 methylation and the equivalent data for patients with normal Dkk-3 gene. CR rates of patients with normal and methylated Dkk-3 gene were 90 and 92%, respectively, accounting for 91% of the overall CR rate. This suggests that methylation of the Dkk-3 gene did not correlate with response to remission induction therapy. However, methylated patients had a
higher relapse rate (57.7% vs 34.7%, \( P = 0.009 \)) than nonmethylated patients. Similar results were obtained in the separate analyses of children (relapse rate: 46% vs 25%, \( P = 0.04 \)) and adults (relapse rate: 71% vs 45%, \( P = 0.03 \)).

We analysed the DFS among patients who achieved CR according to Dkk-3 methylation. Estimated DFS rates at 10 years were 49.8% and 10.5% for normal and methylated patients, respectively (\( P = 0.001 \)) (Figure 2A). Among unmethylated children, the 10 years DFS was 60.6% contrasting with 17.6% for hypermethylated children (\( P = 0.03 \), Figure 2B). Among adult ALL patients, the 9-years DFS was 6.8% for methylated cases and 37.7% for normal cases (\( P = 0.01 \), Figure 2C). The actuarial OS calculated for all leukaemic patients was 45.6% and 15.1% at 11 years for cases with normal and hypermethylated Dkk-3 gene, respectively (\( P = 0.09 \)).

A multivariate analysis of potential prognostic factors (including the type of PETHEMA protocol applied) demonstrated that methylation of the Dkk-3 gene was an independent prognostic factor in predicting DFS in the global series and also in both, childhood and adult ALL (Table 2).

**DISCUSSION**

Mammalian cells have a finite replicative potential *in vitro* and *in vivo* referred to as the Hayflick limit (Hayflick, 1997). Upon reaching this limit, cells cease to divide and undergo genetic, biochemical and morphological changes indicative of ageing, a state referred to as cellular senescence. As these cells do not proliferate, it has been suggested that cellular senescence is a major barrier against cancerous transformation. The ability to overcome senescence and obtain a limitless replicative potential (termed immortalisation), is one of the prerequisites for tumour transformation (Hanahan and Weinberg, 2000). If downregulation of a gene can lead to the immortalisation of cells, the gene may be a candidate of tumour suppressor gene. Recently, a mortalisation-related gene was isolated by the representational difference analysis system with an immortalised cell line and the normal counterpart (Tsuji et al, 2000). A search of this gene revealed that it was identical with the human Dkk-3 gene, an antagonist of the Wnt oncogenic pathway. More recently, it has been reported that Dkk-3 expression was reduced by methylation in several cancer cell lines and primary lung cancers (Kobayashi et al, 2002).

Dkk-3 gene could be a good candidate for epigenetic inactivation in ALL. It is located near the Calcitonin gene, a gene known to be methylated in ALL patients with unfavourable prognosis (Roman-Gomez et al, 2001). However, this gene is generally considered not to be causally related to the malignant process, suggesting that methylation status of the Calcitonin gene region in ALL lymphoblasts may well correspond to that of its neighbouring Dkk-3 gene. In this study, we have identified the Dkk-3 gene as a target gene for methylation and silencing in ALL. Dkk-3 locus methylation was detected in six ALL-derived cell lines and in 33% of tumours at diagnosis, indicating that inactivation of the Dkk-3 gene is a frequent and early event in the process of tumorigenesis in this disease. Methylation of the Dkk-3 promoter region was associated with loss of Dkk-3 gene expression in neoplastic cells and this expression was restored after exposure to the
demethylating agent 5-AzaC, indicating that hypermethylation is a major mechanism by which Dkk-3 expression is silenced in ALL cells. Moreover, a significant concordance between Dkk-3 methylation and Calcitonin methylation was observed, suggesting that Calcitonin methylation is a surrogate for the methylation of the Dkk-3 gene.

What is the functional significance underlying the methylation-mediated transcriptional loss of Dkk-3 in ALL? During replicative senescence altered expression of genes that are involved in cell cycle control and oncogenesis is well documented. A senescence-like arrest can be induced by ectopic expression of p16^{INK4A}, p15^{INK4B}, p21^{CIP} or by DNA-damaging agents that activate p53 (Serrano et al., 1997; Stein et al., 1999). These findings are consistent with a model in which the concurrent induction of a mitogenic stimulus with a forced G_{1} arrest may be sufficient to induce a senescent phenotype. Senescence, much like apoptosis, represents a major barrier to tumorigenesis and it can be achieved by shortened telomeres or conflicting growth signals that forces

Table 1 Clinical characteristics and outcome of 183 ALL patients according to Dkk-3 gene methylation status

| Feature                   | Nonmethylated (n = 123), % | Methylated (n = 60), % | P  |
|---------------------------|---------------------------|------------------------|----|
| Age                       |                           |                        |    |
| Younger than 15 years     | 48                        | 52                     | NS |
| Older than 15 years       | 52                        | 48                     |    |
| Sex                       |                           |                        |    |
| M/F                       | 64/36                     | 72/28                  | NS |
| WBC                       |                           |                        |    |
| Below 50 × 10^9 l^{-1}    | 78                        | 70                     | NS |
| Above 50 × 10^9 l^{-1}    | 22                        | 30                     |    |
| FAB classification        |                           |                        |    |
| L1                        | 38                        | 25                     | NS |
| L2                        | 50                        | 64                     |    |
| L3                        | 12                        | 11                     |    |
| Blast lineage             |                           |                        |    |
| B                         | 88                        | 64                     | NS |
| T                         | 12                        | 36                     |    |
| NCI risk groups           |                           |                        |    |
| Standard                  | 80                        | 65                     | NS |
| Poor                      | 20                        | 35                     |    |
| Pethema risk groups       |                           |                        |    |
| Standard                  | 40                        | 34                     | NS |
| Poor                      | 60                        | 66                     |    |
| Treatment                 |                           |                        |    |
| Pethema 89                | 25                        | 27                     | NS |
| Pethema 93                | 75                        | 73                     |    |
| BMT                       | 19                        | 10                     | NS |
| Best response             |                           |                        |    |
| CR                        | 90                        | 92                     | NS |
| Cyogenetic/molecular      |                           |                        |    |
| Abnormalities             |                           |                        | NS |
| BCR-ABL                   | 17                        | 14                     |    |
| t(1;19)                   | 4                         | 2                      |    |
| 11q23                     | 3                         | 3                      |    |
| c-Myc                     | 6                         | 8                      |    |
| 7q35–Iq11                 | 6                         | 8                      |    |
| Hyperdiploidy             | 9                         | 5                      |    |
| TEL-AML1                  | 5                         | 3                      |    |
| Normal                    | 46                        | 52                     |    |
| Others                    | 3                         | 3                      |    |
| NT                        | 1                         | 2                      |    |
| Relapse                   | 34                        | 57                     | 0.009 |
| Death                     | 41                        | 48                     | NS |

Data are expressed as percentages; WBC = white blood count; FAB = French–American–British; NCI = National Cancer Institute; PETHEMA = Programa para el estudio y tratamiento de las hemopatias malignas; BMT = bone marrow transplantation; CR = complete remission.

Figure 2 Kaplan–Meier survivor function for ALL patients. DFS curves for ALL patients enrolled in this study according to methylation status of the Dkk-3 gene. Solid lines, unmethylated patients; dashed lines, methylated patients.
leukaemogenesis by means of its ability to antagonise Wnt proteins. Wnt pathway plays a crucial role in haematopoietic differentiation (Brandon et al., 2000); and activation of a ‘canonical’ Wnt pathway is associated with several types of human cancers, including B-cell malignancies (Qiang et al., 2003). The ‘transforming activity’ of Wnt is ascribed to the beta-catenin-signalling pathway, which appears to be specifically activated by Wnt7A protein (Sheldahl et al., 1999). Interestingly, Dkk-3 negatively modulates Wnt7A signalling (Caricasole et al., 2003). Therefore, it can be speculated that lack of Dkk-3 expression favours the activation of the most powerful Wnt protein related to the development of cancer.

These findings, together with the prognostic impact of Dkk-3 inactivation in ALL, the general expression of Dkk-3 in normal blood cells, the emerging role of the Dkk-3 gene in human cancer and the observation that transfection of human osteosarcoma Saos-2 cells with Dkk-3 entails a decrease in the proliferative activity are supportive of Dkk-3 inactivation contributing directly to the clinical behaviour of ALL, at least in a subgroup of patients.

In conclusion, our results strongly indicate that the silencing of Dkk-3 expression by aberrant promoter methylation occurs at an early stage of ALL pathogenesis and plays a role in the outcome of the disease.

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Table 2

| Feature                | Univariate analysis | Multivariate analysis |
|------------------------|---------------------|-----------------------|
|                        | P-value             | P-value               |

Global series
Dkk-3 hypermethylation
WBC > 50 000 mm$^3$
BCR-ABL positivity
T phenotype
Age > 15 years
PetHEMA poor risk
Childhood ALL
Dkk-3 hypermethylation
T phenotype
WBC > 50 000 mm$^3$
Adult ALL
WBC > 50 000 mm$^3$
Dkk-3 hypermethylation
BCR-ABL positivity

aberrant cells into a G0-like state (Hanahan and Weinberg, 2000). The genetic changes associated with immortalisation, namely loss of p16, p15, p21 and p53, are among the most common known cancer-related changes, and these genes are frequently down-regulated by methylation in ALL (Garcia-Manero et al., 2002; Roman-Gomez et al., 2003; Agirre et al., 2002). Dkk-3 could function in a similar way, confirming that epigenetic inactivation of genes involved in senescence regulation plays a major role in the pathogenesis of leukaemia. In addition, Dkk-3 could contribute to
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