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Takahiro Sugiyama, Mayda Gursel, Fumihiko Takeshita, Cevayir Coban, Jacqueline Conover, Tsuneyasu Kaisho, Shizuo Akira, Dennis M. Klinman, and Ken J. Ishii

Synthetic immunostimulatory nucleic acids such as CpG DNA are being harnessed therapeutically as vaccine adjuvants, anticancer or antiallergic agents. Efforts to identify nucleic acid-based agents capable of more specifically modulating the immune system are being developed. The current study identifies a novel class of single-stranded oligoribonucleotides (ORN) containing unmethylated CpG motifs and a poly(G) run at the 3' end (CpG ORN) that directly stimulate human CD14^+CD11c^+ monocytes but not dendritic cells or B cells. CpG ORN activate NF-κB and p38 MAPK, resulting in IL-6 and IL-12 production and costimulatory molecule up-regulation but not IFNα. Methylation of cytosine at the 5' portion in core CpG motif abrogates such activation. TLR3, 7, 8, or 9 alone did not confer response to CpG ORN, in contrast to previously reported respective nucleic acid ligands. These data suggest that CpG ORN represent a novel class of synthetic immunostimulatory nucleic acids with distinct target cells, receptors, and functions from that of previously known immunomodulatory nucleic acids. The Journal of Immunology, 2005, 174: 2273–2279.

The innate immune system recognizes specific sequence motifs expressed in the DNA and/or RNA of various pathogens (1–4). For example, recognition of CpG motifs in DNA or dsRNA molecules triggers a strong proinflammatory response via TLRs (5, 6). The immunostimulatory activity of CpG DNA is being harnessed therapeutically to prevent or treat infectious diseases, allergy, cancer, and/or autoimmune diseases (7, 8).

Unmethylated CpG motifs are recognized via TLR9 expressed on certain immune cells (such as B cells, plasmacytoid dendritic cells (pDC), and macrophages) (4, 7). CpG DNA is taken up via an endo/phagocytosis pathway controlled by PI3K (9) and interact with intracellular TLR9 (10). The interaction of CpG DNA with TLR9 triggers the recruitment of the MyD88 adaptor molecule, followed by the activation of IL-1R-associated kinase, TNFR-associated factor 6, and IκB kinase, culminating in the nuclear translocation of NF-κB (5, 11, 12). Such activation by CpG DNA results in the production of proinflammatory cytokines (such as IL-6, IL-12, and IFNα/γ), chemokines (such as MCP-1, IFN-γ-inducible protein 10, MIP-1α/β), and IgG (reviewed in Refs. 4 and 7). Recent studies suggest that CpG oligodeoxynucleotides (ODN) are categorized at least into two groups in both a structural and functional manner (7, 8): one is K (also known as B type) CpG ODN consisted of all phosphorothioate backbone with multiple TCG motifs that preferentially stimulate B cells to proliferate and secrete IL-6 and IgM, and the other is type D CpG ODN (also known as A type) with mixed backbone with one palindromic CpG motifs plus poly(G) run at the 3' end that stimulates pDC to secrete large amount of IFNα, followed by myeloid DC maturation and NK cell activation. These CpG ODN optimized for humans, in contrast to those for mice, are not able to induce a robust amount of IL-12. Since IL-12 is considered to play a critical role in CpG-mediated efficacy in vivo, there is room to improve and/or modify such humanized CpG-based immunotherapeutic nucleic acids.

dsRNA, such as that found during intermediate stages of viral infection, is recognized by the innate immune system through TLR3. dsRNA is a potent stimulator of human monocytes and myeloid DC, inducing the secretion of both IL-12 and IFNα (6, 13). More recently, ssRNA was shown to activate pDC in mice and myeloid DC in humans to produce IFNα and IL-12, respectively (14–17). However, whether the immunomodulatory activity of RNA is sequence specific has not been fully investigated.

The present work examines whether ssRNA have sequence-specific immunomodulatory activity. Results indicate that ssRNA oligonucleotides expressing unmethylated CpG motifs and a poly(G) tail (CpG oligoribonucleotides (ORN)) stimulate CD14^+CD11c^+ monocytes to produce large amounts of IL-12. This activity is distinct from that of DNA-based CpG ODN. CpG ORN also stimulates human PBMC to activate NF-κB and p38 MAPK and to secrete IL-6, similar to the effect of K-type CpG ODN. Activation of cells by CpG ORN was not mediated through either TLR3, 7/8, or 9, the known receptors for dsRNA, ssRNA, or dsRNA, respectively. The activity of CpG ORN was blocked by inhibitors of PI3K. These data suggest that CpG ORN represent novel immunostimulatory agents stimulating human monocytes to produce IL-12 that is distinct from previously known immunostimulatory nucleic acids.
Materials and Methods

Cells and reagents

Normal human PBMC and elutriated monocytes were obtained from the Department of Transfusion Medicine, National Institutes of Health (Bethesda, MD). The purity of elutriated monocytes were 75–90%. Myeloid dendritic cells and pDC were obtained from human monocytes as previously described (18). PBMC were separated by Ficoll-Hypaque density gradient centrifugation as previously described (19). To obtain myeloid dendritic cells, monocytes were cultured for 7 days in the presence of rIL-4 and GM-CSF as previously described (20). In some experiments, pDC were depleted by anti-BDCA-2 Ab using a MACS column according to the manufacturer’s protocol (Milteny Biotec). Cells were cultured in complete medium (RPMI 1640 with 5% FBS) in the presence of various stimuli for 48–72 h at a concentration of 2.5 × 10⁶ cells/ml. HEK-293 cells were purchased from American Type Culture Collection and maintained in DMEM with 10% FBS as described elsewhere (9). Human TLR3 cDNA was a kind gift from Dr. R. Medzhitov (Yale University) and cDNA for human TLR7 and 8 were kind gifts from Dr. B. Beutler (The Scripps Research Institute, La Jolla, CA). TLR3, 7, 8, and 9 were cloned into the pCIneo vector (Invitrogen Life Technologies) and their sequences were confirmed (11). Transfection of HEK-293 cells with these plasmids was performed by using 293fectin (Invitrogen Life Technologies) according to the manufacturer’s recommendation. In some experiments, the PI3K inhibitor wortmannin or the p38 inhibitor SB203580 was used at 1 or 2 μM, respectively. LPS, wortmannin, and SB203580 were purchased from Sigma-Aldrich. dsRNA poly(IC) was purchased from Amersham Pharmacia and R848 was purchased from Invivogen. FuGENE6 was purchased from Roche Molecular Biochemicals.

Synthesis of oligonucleotides

ODN were synthesized at the Center for Biologics Evaluation and Research Core Facility as previously described (21). Some ODN and ORN were purchased from Dharmaco Research. ORN were protected by 2′-O-THP (22) that did not alter their immunostimulatory activity whereas 2′-O-ethyl (22) that did not alter their immunostimulatory activity whereas 2′-O-methyl (2-OME) modification of RNA abrogates such effects (data not shown). ORN were synthesized using either phosphodiester or phosphorothioate linkages. All oligonucleotides contained <0.1 endotoxin unit/ml.

Cytokine ELISA

Cells (2.5 × 10⁶/ml PBMC or elutriated monocytes and 5 × 10⁶/ml HEK-293 cells) were stimulated for 48 h in vitro (24 h for HEK 293 cells). Cell culture supernatants were analyzed for cytokine content by ELISA as described elsewhere (19). Briefly, 96-well Immulon II plates were coated with anti-human IL-6 (clone mAb206; R&D Systems), IL-8 (clone 6217; R&D Systems), IL-12 p70 (clone 20C2; BD Pharmingen), IFN-γ (clone M-701; Endogen), or IFNα (PBL Biomedical Laboratories) in PBS (pH 7.2) for 5 h. The plates were blocked and washed, and then supernatants were added for 2 h at room temperature. The plates were then washed and treated with biotinylated anti-cytokine Ab (IL-6: clone AHC0963; Bio-Rad) using a NeutraPAGE system according to the manufacturer’s protocol (Invitrogen Life Technologies). The membrane was blocked with 5% skim milk and blotted with specific Abs. The membrane-bound Abs were visualized with HRP-conjugated Ab to rabbit IgG using the LumiGLO reagent (Cell Signaling Technology). Anti-phospho-p38, p38, phospho-IκB, and IκB were purchased from Cell Signaling Technology.

Statistical analysis

One-way ANOVA was used to compare groups. Values are expressed as mean ± SD. A p < 0.05 was considered to be significant.

Results

Activity of synthetic RNA containing immunostimulatory CpG motifs

Previous studies established that synthetic DNA-based ODN expressing CpG motifs were immunostimulatory (4, 7). This work examines whether ssRNA oligonucleotides expressing CpG motifs could also activate cells of the immune system. ORN were synthesized using phosphorothioate bases to increase their resistance to nuclease digestion. Initial studies compared the activity of D-type CpG ODN (previously shown to stimulate pDC to produce IFNα) and K-type CpG ODN (previously shown to stimulate B cells to produce IL-6 and proliferate) to their RNA analogues (sequences shown in Table I). Consistent with previous studies, D-type CpG ODN triggered a significant increase in IFNα production by human PBMC, whereas K-type CpG ODN stimulated IL-6 production and proliferation (p < 0.01 for both effects, Fig. 1).

The immunomodulatory properties of D-type CpG ORN, RNA version of D type CpG ODN, were quite distinct from those of either D- or K-type ODN. D-type CpG ORN uniquely induced the production of IL-12 in addition to IL-6 (p < 0.01), yet had no effect on IFNα production and proliferation (Fig. 1a). These immunostimulatory effects were abrogated when the CpG dinucleotides were replaced with GpC (control ORN, GpC), or 5′-methylated C and G (control ORN (methyl C)) (Fig. 1a). None of the K-type CpG ORN was immunostimulatory (Fig. 1a and data not shown).

Table I. Sequences of CpG DNA and CpG RNA

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>ORN sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGTGCACTCATGCAGGAGGGG</td>
<td>GGUGCGACGACGAGGAGGGG</td>
</tr>
<tr>
<td>D-type CpG ODN (D35)</td>
<td>D-type CpG ORN (R35)</td>
</tr>
<tr>
<td>GGTCGATTGAGGGG</td>
<td>GGUGCGACGACGAGGAGGGG</td>
</tr>
<tr>
<td>D-type control ODN (D122)</td>
<td>D-type control ORN (R36,</td>
</tr>
<tr>
<td>ATCGACTCTCGAGCGTTCTC</td>
<td>methylated C)</td>
</tr>
<tr>
<td>K-type CpG ODN (K3)</td>
<td>ATTCGACTCTCGAGGTTCCTC</td>
</tr>
<tr>
<td>K-type control ODN (K10)</td>
<td>ATTCGACTCTCGAGGTTCCTC</td>
</tr>
<tr>
<td>RNA sequence</td>
<td></td>
</tr>
<tr>
<td>D-type CpG ORN (R35)</td>
<td>D-type CpG control ORN (R36,</td>
</tr>
<tr>
<td>GGUGCGACGACGAGGAGGGG</td>
<td>methylated C)</td>
</tr>
<tr>
<td>D-type control ORN (R36, methylated C)</td>
<td></td>
</tr>
<tr>
<td>GGUGCGACGACGAGGAGGGG</td>
<td></td>
</tr>
<tr>
<td>D-type control ODN (R37, GpC)</td>
<td></td>
</tr>
<tr>
<td>Control ORN (R38, poly(A) tail)</td>
<td>GGUGCAUCGUACCGAGAGGGG</td>
</tr>
<tr>
<td>R35 poly(U) (R39, poly-U tail)</td>
<td>GGUGCAUCGUACCGAGAGGGG</td>
</tr>
<tr>
<td>R35 poly(C) (R40, poly(C) tail)</td>
<td>GGUGCAUCGUACCGAGAGGGG</td>
</tr>
<tr>
<td>K-type CpG ORN (R3)</td>
<td>GGUGCAUCGUACCGAGAGGGG</td>
</tr>
</tbody>
</table>

* Nucleotides with uppercase character are phosphorothioate backbone, and those with lowercase letters are phosphodiester backbone. Underlines are active CpG motifs of ODN or ORN.
Monocytes are directly responsive to RNA-based CpG

The cell type(s) responding to D-type CpG ORN was determined by monitoring intracytoplasmic cytokine production by various stimulated cell types. As seen in Fig. 1b, the majority of cells that responded to D CpG ORN by secreting IL-12 and IL-6 were CD14^+ monocytes, while IL-6 production by K-type CpG ODN derived from the CD14^- fraction of PBMC. To further explore the response of monocytes to D-type CpG ORN, their expression of surface markers was examined. As seen in Table II, CD11c^+CD14^- monocytes responded to D-type CpG ORN by up-regulating expression of CD83 and CD86. ORN lacking the critical CpG motif had no such effect. Elutriated monocytes were then stimulated with CpG ORN (Fig. 2a). Consistent with data obtained from unfraccionated PBMC, D type but not control ORN stimulated CD14^-CD11c^- monocytes to produce significant amounts of IL-12 (Fig. 2a). When these elutriated monocytes were analyzed for critical signaling molecules for IL-12, IL-6, and co-stimulatory markers up-regulation, such as NF-κB and p38 MAPK activation (1), CpG ORN but not control ORN (C methylated) induce IκB phosphorylation, its following degradation and p38 phosphorylation within 60 min (Fig. 2b). These data indicate that CD14^-CD11c^- monocytes were major responder cell types in PBMC to activate NF-κB and p38 MAPK, resulting in production of IL-12 and IL-6 and up-regulation of costimulatory markers.

Previous studies showed that DNA-based CpG ODN act on B cells and pDC rather than monocytes. However, factors produced by pDC may indirectly stimulate monocytes in the same cultures (23). We therefore examined whether CpG ORN were acting directly on monocytes or through pDC intermediates. PBMC were elutriated to generate highly enriched monocyte preparations, and these preparations were further purified by specific depletion of pDC. Consistent with previous reports, elimination of pDC abrogated the ability of D-type ODN to stimulate the rest of the cells to produce IFNγ (Fig. 2c). In contrast, removal of pDC had no impact on the ability of D-type CpG ORN to stimulate highly purified monocytes to produce IL-12 or IL-6 (Fig. 2c). These findings suggest that unlike their CpG-containing DNA counterparts, CpG-containing ORN act directly on monocytes.

**Immunostimulatory RNA requires an unmethylated CpG motif and a 3’ poly(G) tail**

The influence of sequence and structure on the immunostimulatory activity of D-type ORN was examined. Stimulation was abrogated when the central CG was flipped to GC or the C methylated (Fig. 3a). Stimulation was also reduced by substitutions in the 5’ or 3’ region immediately flanking the CG dinucleotides. Specifically, optimal stimulation was elicited by a 3’ AU and reduced by replacements by AA or UA. The 5’ AU was less critical to D-type CpG ORN stimulation (Fig. 3b and data not shown).

Whereas D-type CpG ODN require that the bases flanking the central CpG motif form a palindrome (19), no such structural requirement was observed for D-type CpG ORN (Fig. 3a). However, both D-type ODN and ORN required a poly(G) run at their 3’ end to mediate maximal immune stimulation (19) (Fig. 3b).

The effect of the nucleotide backbone on immunostimulatory activity of CpG ORN was then examined. D-type CpG ODN are optimally active when both termini are composed of phosphorothioate linkers and the central region is phosphodiester (19). In contrast, pure phosphorothioate CpG ORN were strongly stimulatory (Fig. 3c). Indeed, the level of immune stimulation fell as phosphodiester linkages were added to the central region. This may

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**Table II. CpG RNA increases costimulatory molecule or activation marker expression on immune cells**

<table>
<thead>
<tr>
<th></th>
<th>CD11c/CD83 (MFI)</th>
<th>CD11c/CD86 (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-type CpG ORN</td>
<td>6.1 ± 2.9*</td>
<td>6.3 ± 2.4*</td>
</tr>
<tr>
<td>(R35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-type control ORN</td>
<td>1.4 ± 0.8</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>(R37, GpC)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fold increase (media = 1, *, P < 0.05.)
MFI, mean fluorescence intensity.

---

**FIGURE 1.** Stimulation of human PBMC by CpG ORN. Human PBMC were stimulated in vitro with synthetic ssDNA or ssRNA containing K- or D-type CpG motifs for 48 h. a, The production of IL-6, IL-12, and IFNγ and proliferation were measured by ELISA as described in Materials and Methods. Each open circle represents results from a single donor, while the bar represents the averages (geometric mean) of all values (*, p < 0.01 vs medium). b, Cell surface expression of CD14 and intracellular cytokine production (IL-6 and IL-12) were measured by flow cytometry as described in Materials and Methods. Numbers show the percentage of cells in the gated area. Results are representative of two individual experiments.
reflect increased sensitivity to nuclease degradation (rather than any effect on structure) since pure phosphodiester CpG ORN were stimulatory if introduced directly into cells by transfection (Fig. 3).

These findings suggest that structural requirements for immunologically active D-type ORN are distinct in terms of base, backbone, and flanking sequences to those for D-type ODN, with the exception that both require the presence of an unmethylated CpG motif and a 3’ poly(G) tail (19).

TLRs 3, 7, 8, or 9 do not confer recognition of CpG ORN

TLRs 3, 7, 8, and 9 have been shown to recognize distinct structures expressed by nucleic acids (or their analogues) (3). Thus, transfection of a cell line lacking these genes (such as HEK-293) with genes encoding these specific receptors confers responsiveness to the relevant TLR ligand (6, 11, 24). The ability of CpG ORN to interact with these TLRs was examined by transfecting HEK-293 cells with TLR3, 7, 8, or 9. Although such TLR transfections resulted in successful recognition of respective known ligands, none of the transfected cells responded to D-type ORN (Fig. 4).

Discussion

The host’s innate immune system recognizes and responds to DNA and RNA expressed by infectious pathogens. Accumulating evidence suggests that most but not all of this recognition is mediated by TLRs. For example, TLR9 is critical for the recognition of single-stranded CpG DNA (4, 7), while dsDNA up-regulates MHC-related genes through TLR-independent pathways (K. J. Ishii, unpublished data), which was independent of the existence of CpG motifs within DNA sequences (26, 27). Similarly, dsRNA stimulates immune and nonimmune cells via both TLR3 and protein kinase, dsRNA dependent (6, 28). ssRNA was recently shown to stimulate dendritic cells via TLR7 or 8 (14–17). Heil et al. (15) demonstrated that GU-rich ssRNA stimulated pDC via TLR7 in mice and possibly TLR8 in humans. Diebold et al. (14) reveal that the nucleotides preference of ssRNA-mediated pDC activation was attributed to poly(U) but not poly(G), poly(C), or poly(A). Koski et al. (16) have shown that human myeloid dendritic cells are activated by uridine-rich ssRNA derived from bacteria without the poly(A) tail but not from vertebra mRNA containing the poly(A) tail (16).

The current work evaluated whether ssRNA-containing CpG motifs was immunostimulatory. Results obtained from >20 healthy donors’ PBMC demonstrate that CpG ORN can stimulate human PBMC to produce significant amounts of IL-12 and IL-6.
and to induce the up-regulation of costimulatory molecules (Fig. 1 and Table II). This activation requires CpG motifs that are characteristic of D-type CpG ODN. Optimal D-type CpG ODN contain purine-pyrimidine-CG-purine-pyrimidine as a core sequence, and Phosphorothioate linkages are shown as uppercase letters, while the others are phosphodiester.

FIGURE 3. Structural elements required for immunostimulatory activity of CpG ODN. Human elutriated monocytes were stimulated with various ORN or ODN (3 μM) for 48 h and the production of IL-12, IFNα, or IL-6 was measured by ELISA. a, The hexametric CpG motifs in D-type ORN (R35) were modified as indicated, while the other nucleotide sequences are identical to R35 (see Table I). b, The hexametric 3′ poly(G) sequences of R35 were replaced with either poly(A), poly(C), or poly(U). -CG- indicates that CG dinucleotides in CpG motifs of R35 are flipped to GC. -5mCG- indicates that the 5′ portion of cytosine in the CG dinucleotides was methylated. The other nucleotide sequences of ORN are identical to R35 (see Table I). c, Backbone of R35 (see Table I) was modified.

FIGURE 4. Involvement of TLRs in CpG ORN-induced immune responses. HEK-293 cells (5 × 10⁴) were transfected with plasmids encoding human TLR3, 7, 8, or 9 (1 μg) for 24 h. a, The cells were then stimulated with CpG ODN (K3, 1 μM), LPS (100 ng/ml), dsRNA (20 μg/ml), R848 (1 μM), or D-type CpG ORN (R35, 1 μM) for 24 h. The TLR-transfected cells were stimulated with CpG ORN mixed with FuGENE6 (FuG) as indicated. The concentration of IL-8 in culture supernatants was measured by ELISA as described in Materials and Methods.
a poly(G) tail at the 3’ end (19). Similarly, an unmethylated CG dinucleotide flanked by AU and a 3’ poly(G) tail was required for CpG ORN to induce IL-12 production by monocytes (Fig. 3, a and b).

Unlike D-type CpG ODN, CpG ORN were active when the backbone was entirely phosphorothioate. This may reflect the ability of phosphorothioate ODN to resist nuclease degradation, since phosphodiester ORN were as stimulatory if transfected directly into target cells (Fig. 3, c and d). Of considerable interest, both the target population of CpG ORN and the nature of the induced response were quite different from that described for CpG ODN. CpG ORN directly stimulated CD14+ CD11c+ monocytes to secrete IL-12, yet had no effect on T cells, B cells, or pDC in the productions of cytokines and chemokines and the up-regulation of MHC and costimulatory molecules as far as we examined (Figs. 1b and 2r; data not shown). In addition, D-type ORN did not stimulate PBMC or purified pDC to produce IFNγ, whereas D-type CpG ODN directly stimulate pDC to produce large amounts of IFNγ. Although cells that respond to CpG ORN are quite distinct from those of CpG ODN, CpG ORN directly activate CD14+ CD11c+ monocytes and indirectly stimulate NK cells to produce IFN-γ mediated through IL-12, to a similar extent as that induced by CpG ODN (Table II and T. Sugiyama and K. J. Ishii, unpublished results).

These distinct immunostimulatory activities of CpG ORN led us to further study whether CpG ORN utilize TLRs. If so, which TLR is involved in CpG ORN-induced immune activation? Previous reports have shown that human monocytes express mRNA of TLR8, but not TLR3, 7, or 9 (29), suggesting that TLR8 might be the receptor for CpG ORN (15, 16). Yet HEK-293 cells transfected with TLR8 did not respond to D-type CpG ORN (Fig. 4). This finding suggests that either TLR8 is not involved in the recognition of CpG ORN or that some coreceptor(s), adaptor(s), and/or signaling molecule(s) is additionally needed for CpG ORN-mediated activation even if TLR(s) is involved (11, 30).

Significance of our finding in physiological conditions is yet unclear. Unmethylated CpG motifs in genome are observed more frequently in bacterial and certain viral DNA than vertebran DNA, thereby being recognized by the innate immune system via TLR9 (3, 4, 7, 8). In contrast, to our knowledge, TLR8, but not TLR3, 7, or 9 (29), suggesting that TLR8 might be the receptor for CpG ORN (15, 16). Yet HEK-293 cells transfected with TLR8 did not respond to D-type CpG ORN (Fig. 4). This finding suggests that either TLR8 is not involved in the recognition of CpG ORN or that some coreceptor(s), adaptor(s), and/or signaling molecule(s) is additionally needed for CpG ORN-mediated activation even if TLR(s) is involved (11, 30).

In summary, the current work demonstrates that ssRNA-containing CpG motifs directly stimulate human monocytes to activate NF-κB and p38 MAPK, resulting in the productions of IL-6 and IL-12 and up-regulation of costimulatory markers such as CD86. Their activity and mode of recognition is distinct from that of CpG ODN, dsRNA, or GU-rich ssRNA. Additional study is required to determine whether the unique effects of CpG ORN can be used therapeutically, but the importance of IL-12 in protective immune response in infection, allergy, or cancer suggests that the capacity to selectively induce these cytokines may be of benefit in the development of vaccine adjuvants, immunoprotective agents for infection, and antiallergic or anticancer agents.

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