MADM, a Novel Adaptor Protein That Mediates Phosphorylation of the 14-3-3 Binding Site of Myeloid Leukemia Factor 1*

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A yeast two-hybrid screen was conducted to identify binding partners of Mlf1, an oncoprotein recently identified in a translocation with nucleophosmin that causes acute myeloid leukemia. Two proteins isolated in this screen were 14-3-3 and a novel adaptor, Madm. Mlf1 contains a classic RSXSP sequence for 14-3-3 binding and is associated with 14-3-3 via this phosphorylated motif. Madm co-immunoprecipitated with Mlf1 and localized exclusively in the nucleus. In addition, Madm recruited a serine kinase, which phosphorylated both Madm and Mlf1 including the RSXSP motif. In contrast to wild-type Mlf1, the oncogenic fusion protein nucleophosmin (NPM)-MLF1 did not bind 14-3-3, had altered Madm binding, and localized exclusively in the nucleus. Ectopic expression of Madm in M1 myeloid cells suppressed cytokine-induced differentiation unlike Mlf1, which promotes maturation. Because the Mlf1 binding region of Madm and its own dimerization domain overlapped, the levels of Madm and Mlf1 may affect complex formation and regulate differentiation. In summary, this study has identified two partner proteins of Mlf1 that may influence its subcellular localization and biological function.

Myeloid leukemia factor 1 (MLF1) is a recently described oncogene involved in acute myeloid leukemia and myelodysplastic syndrome. It was initially identified in a t(3;5)(q25.1; q34) translocation between MLF1 on chromosome 3 and nucleophosmin (NPM) on chromosome 5, which generates the chimeric molecule NPM-MLF1 (1). Significantly, overexpression of wild-type MLF1 in acute myeloid leukemias, not involving the t(3;5), is associated with progression toward a malignant phenotype (2).

The murine homologue of MLF1 (Mlf1) (3) was isolated independently as a gene expressed when J2E erythroleukemia cells (4) undergo a spontaneous phenotypic change to display morphological and biochemical features of monocytoid cells (5); therefore, the gene was termed Hls7 for hemopoietic lineage switch gene 7 (6). Significantly, enforced expression of Mlf1/Hls7 in the parental erythroleukemia cells recapitulated the original phenotypic change and prevented erythropoietin-induced maturation (6). In addition, ectopic expression of Mlf1/Hls7 in normal hemopoietic progenitors enhanced myeloid colony formation at the expense of erythroid colonies. From these studies, it was concluded that Mlf1 is normally involved in the phenotypic determination of hemopoietic cells and that its dysregulation is important in leukemogenesis.

Analysis of the Mlf1 sequence identified no recognizable motifs or domains, except for a characteristic RSXSP binding site for 14-3-3 proteins (3, 6). Proteins that associate with 14-3-3 family members are phosphorylated on serine residues within the RSXSP motif (7, 8). This serine phosphorylation appears essential for 14-3-3 binding, although other non-canonical binding sites have also been identified in molecules such as Cbl (9) and p53 (10). The 14-3-3 family of proteins is expressed in a broad range of organisms and is highly conserved throughout evolution. These molecules bind and influence the activity of numerous diverse proteins, including Raf, BAD, Cdc25c, p53, Cbl, Bcr, and Bcr-Abl (10–15); as a consequence, they have been implicated in regulating the onset of cell division, apoptosis, and differentiation. 14-3-3 proteins control the activity of their partner molecules, in part, by sequestering them from their site of action and by serving as bridging molecules that promote dimerization of associated proteins (16).

In an attempt to decipher the biological function of Mlf1, a yeast two-hybrid screen was conducted to identify molecules that associate with the protein. Predictably, a 14-3-3 family member (14-3-3z) was shown to associate with Mlf1 in this screen. In addition, a novel adaptor molecule (Madm, MLF1-adaptor molecule) was isolated. Madms associates with a kinase that phosphorylates serine residues in Madm itself and the
**RESULTS**

**Mlf1 Associates with 14-3-3-ζ—**In an attempt to identify proteins that interact with Mlf1, a yeast two-hybrid screen was performed. Table I shows that Mlf1 interacted with numerous molecules, including a member of the 14-3-3 family of proteins viz 14-3-3ζ, which was isolated as two separate clones. Other molecules that associated with Mlf1 in this screen included eukaryotic translation initiation factor 3 (eIF3, p42 subunit), protein inhibitor of neuronal nitric-oxide synthase (PIN, also known as dynein light chain, LC8), calycin-binding protein, nucleolin, and several uncharacterized proteins; each of these were identified once in the screen.

To confirm the interaction between Mlf1 and 14-3-3ζ biochemically, COS cells were transfected with an Mlf1-expressing construct since endogenous Mlf1 protein expression is below the limits of detection. Fig. 1A shows that when lysates were immunoprecipitated using anti-14-3-3ζ antibodies, Mlf1 specifically co-purified with endogenous 14-3-3ζ. In addition, Mlf1 co-immunoprecipitated with 14-3-3ζ in J2E erythroleukemia cells expressing retrovirally driven Mlf1 (data not shown). These results corroborated the interaction between Mlf1 and 14-3-3ζ in the yeast two-hybrid screen.

Both murine and human MLF1 contain a classic RXXSRXP motif for 14-3-3 binding (1, 3, 6). To determine if this region mediates the interaction between MLF1 and 14-3-3ζ, two peptides spanning this motif were generated (MRQMRIRSFSFEPFGRDL1)1 with serine 34 either phosphorylated or non-phosphorylated. Data displayed in Fig. 1B show that endogenous and recombinant 14-3-3ζ associated with the phosphorylated peptide only. Moreover, only the phosphorylated peptide was able to disrupt the MLF1/14-3-3ζ complex (Fig. 1C). Taken together, these results demonstrate that MLF1 and 14-3-3ζ associate via the RXXSRXP motif of MLF1 and that phosphorylation is essential for this interaction.

The binding of 14-3-3ζ to the fusion protein NP-M-MLF1 was also investigated. Immunoprecipitation experiments from COS cells expressing FLAG-tagged NP-M-MLF1 demonstrated that
NPM-MLF1 did not bind 14-3-3\(\zeta\) (Fig. 1D) or that the interaction was extremely weak. Therefore, despite retaining the RSX-SXP motif, the leukemogenic fusion protein NPM-MLF1 differed biochemically from the wild-type MLF1. These data suggest that the inability of NPM-MLF1 to associate with 14-3-3 may occur due to conformational changes produced by the fusion of NPM to MLF1.

**Mlf1 Associates with a Novel Adaptor Protein**—One of the novel clones that associated with Mlf1 in the yeast two-hybrid screen (Clone M44) contained a 220-bp cDNA fragment, which had no homology to sequences in the database at the time of isolation and was named Madm for Mlf1-adaptor molecule. This fragment was used to screen an EML C.1 cDNA library, and a 2153-bp cDNA was isolated. An open reading frame of 1608 bp was identified, encoding a putative 535-amino acid protein with a predicted molecular mass of 60 kDa (Fig. 2A). The probable initiating ATG was in the characteristic context for a translation start site (25).

A comparison of the amino acid sequence encoded by Madm using the Swiss-Prot data base revealed similarities with a
FIG. 2. M44 cDNA and protein sequence. A, the nucleotide sequence of murine Madm cDNA is shown with the predicted amino acids indicated above. Nucleotides are numbered on the left and amino acids on the right. The in-frame termination codons in the 5' untranslated region are underlined. The putative kinase domain is in black and the nuclear export sequence (NES, amino acids 121–129) and nuclear localization sequence (NLS, amino acids 163–181) are light gray. The boxed sequence indicates the region identified in the Mlf1 yeast two-hybrid screen. Dashed lines indicate the two putative nuclear receptor-binding motifs (consensus, LXXLL). B, schematic representation of the Madm protein shows the potential SH2-binding region, the kinase-like domain, and the Mlf1-binding domain. The genomic organization of the Madm gene is shown below with 18 exons identified by boxes.
variety of protein kinases, and a consensus kinase domain was identified (26). However, the conserved ATP-binding motif (GXXGXXG), found in conventional protein kinases (27), was not present in this protein (Fig. 2). Intriguingly, a bipartite nuclear localization signal (NLS) was present within the kinase domain, as well as a potential nuclear export signal (NES) (Fig. 2), indicating that the protein may shuttle between the nucleus and cytoplasm. Moreover, Madm contains several potential phosphorylation sites in the vicinity of both the NLS and NES. These include protein kinase C phosphorylation sites at Ser102, Ser157, and Thr 165, as well as a casein kinase II phosphorylation site at Thr171, suggesting that phosphorylation may play a role in regulating Madm subcellular localization. In addition, the N-terminal region of Madm is rich in glutamic acid and serine residues, which could potentially bind SH2 modules in a phosphotyrosine-independent manner (28).

Northern blotting analysis and whole mount in situ hybridization demonstrated that Madm was expressed in all murine tissues and hemopoietic cell lines studied; moreover, the levels of mRNA were comparable in all samples analyzed (data not shown). Isolation of murine genomic clones revealed that the gene contains 18 exons, spanning 11 kb (Fig. 2B). Examination of the promoter region identified possible transcription factor binding sites typical of a ubiquitously expressed gene (data not shown).

Sequence comparisons of Madm shown in Fig. 3 revealed that it had 51% amino acid identity with an uncharacterized protein from Drosophila melanogaster (AF145690) and 36% identity with a predicted protein from Caenorhabditis elegans (C599887), suggesting evolutionary conservation. Recently, a human homologue of Madm was submitted to GenBank as nuclear receptor binding protein (NM-013392) because it contained two LXXLL putative nuclear receptor binding motifs (29). The human clone contains an open reading frame of iden-
FIG. 4. Madm and Mlf1 co-immunoprecipitate and co-localize. A, lysates from COS cells transfected with Mlf1 and/or Myc-tagged Madm were immunoprecipitated (IP) with anti-Myc antibodies and immunoblotted (IB) with antibodies to Madm or Mlf1. B, nuclear and cytoplasmic localization of Madm in transfected COS cells expressing green fluorescent protein-Madm (green). Nuclei (blue) were stained with Hoescht 33258. C, COS cells transfected with Madm were separated into nuclear and cytoplasmic fractions and then immunoblotted (IB) with antibodies against Madm, nucleolin (nuclear marker), and 14-3-3ζ (cytoplasmic marker). D, COS cells were co-transfected with Madm and Mlf1. Madm was detected using anti-Myc antibodies and a green fluorescent anti-mouse secondary antibody, while Mlf1 was detected with anti-Mlf1 and a red fluorescent anti-rabbit secondary antibody. Nuclei are shown in blue.
tical size to Madm and shares 98% amino acid identity (Fig. 3).

Madm and Mlf1 Co-immunoprecipitate and Co-localize in Cells—To investigate the interaction between Madm and Mlf1 further, COS cells were transfected with plasmids expressing full-length Mlf1 and Myc-tagged Madm. Fig. 4A shows a 65-kDa band for tagged Madm, which is close to the predicted molecular mass of 60 kDa. Significantly, transfection of both Madm and Mlf1 resulted in co-immunoprecipitation of the two proteins. A reciprocal co-immunoprecipitation in cells expressing HA-tagged Mlf1 and full-length Madm supported these observations (data not shown). These data confirm the association between Madm and Mlf1 identified by yeast two-hybrid analysis.

Mlf1 has been shown previously to be localized primarily in the cytoplasm, but also within nuclear spots (1, 6). Confocal microscopy was employed to determine the subcellular localization of Madm and whether this overlapped with Mlf1. Fig. 4B shows that Madm could be detected mainly in the cytoplasm of transfected COS cells, with some expression in the nucleus. Biochemical analyses of nuclear and cytosolic fractions confirmed that Madm was present in both compartments (Fig. 4C). Co-localization with Mlf1 was observed primarily in the cytoplasm, especially in the perinuclear region including centroosomes (Fig. 4D). Therefore, Madm and Mlf1 associate in the yeast two-hybrid system, co-immunoprecipitate, and share subcellular locations.

Madm and Mlf1 Binding Domains—To characterize the domains required for the Madm and Mlf1 interaction, a series of deletion mutants was generated and tested in both yeast two-hybrid and co-immunoprecipitation studies. Fig. 5A summarizes the data from yeast two-hybrid experiments and shows that amino acids 406–479 of Madm, encoded by the cDNA fragment originally identified in the yeast two-hybrid screen, were able to bind Mlf1. Similarly, deletion mutants of Mlf1 were examined for their Madm-binding ability in yeast. Wild-type Mlf1 and a C-terminal deletion to residue 227, bound Madm (Fig. 5B). The inability of the 1–163 mutant to bind Madm suggested that amino acids between 163 and 227 were important for this association. However, a mutant consisting of amino acids 122–267 did not bind Madm, indicating that regions in the N terminus of Mlf1 were also required for interaction. Furthermore, the 39–267 mutant was able to bind Madm, whereas the 82–267 mutant did not. Collectively these data indicate that a combination of residues between amino acids 39–82 and 163–227 are involved in the Madm association. These findings were confirmed by co-immunoprecipitation from
Fig. 6. Madm, Mlf1, and NPM-MLF1 are phosphorylated on serine residues. A, full-length Madm (Madm FL) and deletion mutants lacking the kinase-like domain (MadmΔkinase), the 57, 129, or 209 C-terminal residues (MadmΔC57, MadmΔC129, or MadmΔC209) were immunoprecipitated (IP) and then subjected to in vitro kinase assays. The fold increase in myelin basic protein (MBP; 1 µg) phosphorylation is shown below. B, COS cells transfected with Madm and/or Mlf1 were lysed, Madm immunoprecipitated (IP) with anti-Myc antibodies, and then subjected to a kinase assay. C, COS cells transfected with Madm, Mlf1, or NPM-MLF1 were phosphorylated in vivo with H32PO4 and protein immunoprecipitated as shown. D, phosphoamino acid analysis of in vivo phosphorylated Madm, Mlf1, and NPM-MLF1. The position of ninhydrin-stained amino acid standards is shown by circles. E, peptides bearing the 14-3-3-binding motif of Mlf1 were incubated with Myc-tagged Madm/kinase complex immunoprecipitated with anti-Myc antibodies before being subjected to a kinase assay.
COS cells where expression of the mutant proteins was demonstrated.

Binding of the fusion molecule NPM-MLF1 to Madm was also ascertained using co-immunoprecipitation experiments from COS cells. The full-length NPM-MLF1 and a C-terminally-truncated protein were able to interact with Madm (Fig. 5C). In addition, deletion of amino acids 281–362 of NPM-MLF1 (corresponding to amino acids 121–202 of MLF1) retained Madm binding; however, removal of the N-terminal residues of MLF1 present in the fusion protein (corresponding to amino acids 17–120 in the normal MLF1 protein) prevented binding. From these data, it appears that Mlf1 and NPM-MLF1 share an N-terminal region that interacts with Madm, but that the second, C-terminal, region identified in MLF1 may be dispensable for NPM-MLF1 interaction with Madm.

Madm Associates with a Serine Kinase That Phosphorylates Madm and Mlf1—Although Madm has a consensus kinase domain, it lacks a typical ATP-binding motif (Fig. 2); it was, therefore, important to determine whether Madm was a genuine kinase. When bacterially produced Madm was subjected to an autokine assay there was no protein phosphorylation, indicating an absence of catalytic activity (data not shown). This observation was also supported by the inability of mammalian-expressed Madm to autophosphorylate under the reducing conditions of in-gel kinase assays. However, when immunoprecipitated from transfected COS cells, full-length Madm and several deletion mutants, including a Madm construct lacking the kinase-like domain, were phosphorylated in vitro kinase assays (Fig. 6A). These data suggested that a separate kinase was precipitating with, and phosphorylating, Madm. Indeed, the phosphorylation of myelin basic protein was increased 3–7-fold when added to kinase assays containing Madm immunoprecipitates (Fig. 6A). In addition, the kinase recruited by Madm was able to phosphorylate co-precipitated Mlf1 (Fig. 6B).

The in vivo phosphorylation status of Madm, Mlf1, and the fusion NPM-MLF1 were investigated in transfected COS cells. When the cells were labeled with [32P]phosphoric acid, all three proteins were phosphorylated (Fig. 6C). Phosphoamino acid analysis of Madm, Mlf1, and NPM-MLF1, following in vivo phosphorylation, revealed that these proteins were phosphorylated on serine residues (Fig. 6D). Preliminary studies have excluded several well characterized kinases as the Madm-associated kinase, including protein kinase A, protein kinase C, Akt, and calcium/calmodulin-dependent kinase. It was concluded from these experiments that Madm recruits a serine kinase that phosphorylates both Madm and Mlf1.

Madm-associated Kinase Phosphorylates 14-3-3 Binding Motifs—As MLF1 contains a consensus 14-3-3 binding site and Madm binds a serine kinase, we postulated that Madm may mediate phosphorylation of the RSXSXP motif in MLF1. To determine whether the Madm/kinase complex was capable of phosphorylating this domain, the peptide 26MRQMIRSFSEPFGRDL41 was used in an in vitro kinase assay. Fig. 6E shows that in addition to the phosphorylation of Madm seen in transfected COS cells, a concentration-dependent phosphorylation of

Fig. 7. Madm forms dimers via a C-terminal region. A, COS cells transfected with vector, Myc-tagged and/or HA-tagged Madm were lysed and immunoblotted (IB) with anti-Myc or anti-HA antibodies (upper panels). Dimerization was demonstrated by Madm immunoprecipitation (IP) using anti-Myc antibodies, followed by immunoblotting with anti-HA antibodies (lower panel). B, deletion mutants of Madm were analyzed for their ability to bind full-length Madm by reporter gene activation in yeast.
The MLF1 peptide bearing the 14-3-3 binding site occurred. Although a limited amount of peptide phosphorylation by endogenous kinase(s) was observed in non-transfected COS cells, phosphorylated peptide levels were 4–6-fold higher with the exogenous Madm complex. These data demonstrate that Madm binds a kinase capable of phosphorylating the RSXSP motif of MLF1. Phosphorylation of the RSXSP motif of Raf1 (8) was also observed with the Madm complex but not the non-canonical 14-3-3 binding site of Cbl (14) (data not shown).

Madm Dimerizes and Influences Myeloid Differentiation—Preliminary experiments with the yeast two-hybrid system indicated that Madm was capable of homodimerization (data not shown). To confirm that Madm could also dimerize in mammalian cells, Madm constructs tagged with either Myc or HA were co-transfected into COS cells. Co-immunoprecipitation with anti-Myc or anti-HA antibodies, followed by appropriate immunoblotting, confirmed that Madm was indeed able to self-associate (Fig. 7A). Data presented in Fig. 7B indicate that the region of Madm involved in homodimerization overlapped with the MLF1-binding domain. This observation sug-
gests that Madm and Mlf1 may form multimeric complexes, or alternatively, compete for binding sites.

We have previously shown that Mlf1 overexpression in M1 monoblastoid cells enhances differentiation induced by LIF, as measured by surface Cd11b expression (6). To determine the impact of Madm on myeloid maturation, the full-length cDNA was introduced into M1 cells using the MSCV retroviral vector. Despite large amounts of viral RNA being generated, Madm protein levels increased no more than 50–80% (Fig. 8, A and B). However, in marked contrast with M1 cells overexpressing Mlf1 (Ref. 6 and data not shown), all clones expressing exogenous Madm displayed lower Cd11b levels in the absence of LIF or when exposed to suboptimal concentrations of the cytokine (Fig. 8C). Some clones persisted with lower Cd11b expression even at higher concentrations of LIF. These results demonstrate that modest alterations to the concentration of Madm can influence the maturation of M1 cells and that increasing the intracellular content of Madm had the opposite effect to Mlf1 on differentiation.

**DISCUSSION**

Mlf1 is a recently described oncprotein (1). It is able to influence hematopoietic lineage commitment and reprogram erythroleukemic cells to develop a monoblastoid phenotype (6). The importance of Mlf1 is highlighted by (i) the t(6;3) translocation, which exposes the leukemogenic potential of the molecule, and (ii) overexpression of wild-type Mlf1 in patients with acute myeloid leukemia, which correlates with poor prognosis and reduced survival (1, 2).

In this manuscript we describe two molecules that interact with Mlf1, viz. 14-3-3 and Madm. The identification of 14-3-3 as an Mlf1 partner protein in a yeast two-hybrid screen and confirmation by co-immunoprecipitation was not surprising as the only recognizable domain in Mlf1 is the 14-3-3 binding motif, RXSXXP (3, 6). It is noteworthy that Mlf1 is serine-phosphorylated in vivo and the phosphorylated motif associated with 14-3-3, but not the unphosphorylated form. 14-3-3 proteins have been implicated in many diverse functions, and their role in promoting cytoplasmic localization of some partner proteins is well documented, e.g. phosphorylation of cdc25c by Chk1 results in 14-3-3 binding, which masks an NLS and retains cdc25c in the cytoplasm (30, 31). It is possible, likewise, that phosphorylation of the RXSXXP motif in Mlf1 promotes 14-3-3 binding, thereby sequestering the molecule in the cytoplasm and restricting its access to the nucleus. This model is consistent with primary localization of wild-type Mlf1 in the cytoplasm, with small amounts detected in punctate nuclear bodies (1, 6, 32). Importantly, although the NPM-MLF1 fusion protein is phosphorylated in vivo, it no longer binds 14-3-3. Because the fusion junction is only 13 residues from the RXSXP motif, it is conceivable that structural alterations may inhibit the binding of 14-3-3.

Madm is a novel adaptor molecule for Mlf1. The co-localization of Madm and Mlf1 suggests that these two proteins are intimately associated, especially in the perinuclear area. Although Madm has a clearly defined kinase domain, it does not contain a characteristic ATP-binding region (29). While a number of kinases that lack this domain have been identified, including Chk1 and cdc7 (33, 34), our data indicate that Madm does not possess the catalytic activity of a kinase. Indeed, Hooper et al. considered that the human homologue of Madm was unlikely to possess functional kinase activity because it lacked 7 of the 15 highly conserved kinase domain residues (29). However, Madm does recruit a serine kinase that phosphorylates both Madm and Mlf1. Specifically, the Madm-associated kinase was able to phosphorylate the RXSXXP motif of Mlf1. Phosphorylation of the comparable motif in Raf1 by the Madm-kinase complex indicates that Madm may act as a more global adaptor involved in phosphorylating other 14-3-3 interacting proteins. The extremely high degree of Madm conservation between mouse and human (98% identity) is noteworthy. In addition, Mlf1 shares 79% identity with the human homolog, and 14-3-3 proteins are highly conserved among all species (35). This may indicate that Madm-Mlf1–14-3-3 complexes serve evolutionarily conserved functions.

The opposing effects of Madm and Mlf1 on M1 cell differentiation are significant. Whereas expression of exogenous Mlf1 potentiated M1 maturation (6), differentiation was inhibited when the level of Madm was raised only 50–80%. This modest increase in the concentration of Madm had a marked impact on the capacity of myeloid cells to mature and was contrary to the effects of Mlf1. As the dimerization domain of Madm overlapped with the Mlf1-binding region, altering the Madm/Mlf1 ratio is likely to affect complex formation between these proteins. The composition of these complexes may, in turn, dictate the progress of terminal differentiation. It is unlikely that these effects were solely due to gross mRNA overexpression because equivalent amounts of retroviral transcripts were detected in M1 cells expressing the vector alone (data not shown). Madm contains a bipartite NLS, as well as an NES. Confocal microscopy and biochemical analyses revealed that Madm was present in both the cytoplasm and nucleus, indicating it may shuttle between the compartments. It is interesting to note that Madm has a potential phosphorylation site within the NLS, which could possibly regulate transport to the nucleus as seen with IFI-16 (36) and APC (20). The presence of two nuclear receptor binding motifs introduces the tantalizing prospect of specific functions for Madm within the nucleus.

There are several possible reasons for the leukemogenic actions of NPM-MLF1. One likely explanation is the inappropriate cellular compartment occupied by the fusion protein (1, 32). Unlike wild-type Mlf1, NPM-MLF1 is found almost exclusively in the nucleus. Data presented in this manuscript demonstrate that NPM-MLF1 is unable to associate with 14-3-3 and that the C-terminal Madm-binding domain is compromised. These observations suggest that the NPM portion of the fusion protein sterically hinders 14-3-3 binding and interferes with the Madm interaction. As a consequence, NPM-MLF1 may not be sequestered in the cytoplasm and is transported into the nucleus by the NLS within NPM.

We postulate that the two partner proteins of Mlf1 described in this study play a pivotal role in regulating its subcellular localization and potentially its function. Madm recruits a serine kinase that phosphorylates the RXSXXP motif of Mlf1, thereby promoting 14-3-3 binding. Complexes involving Mlf1, Madm, and 14-3-3 are likely to influence the distribution of these proteins in the cell and affect their ability to differentiate.

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