Enzyme deficiency in the salvage pathway of deoxyribonucleotide synthesis in mitochondria can cause mtDNA depletion syndromes. We have identified a human mitochondrial UMP-CMP kinase (UMP-CMPK, cytidylate kinase; EC 2.7.4.14), designated as UMP-CMP kinase 2 (UMP-CMPK2). The C-terminal domain of this 449-amino acid protein contains all consensus motifs of a nucleoside monophosphate kinase. Phylogenetic analysis showed that UMP-CMPK2 belonged to a novel nucleoside monophosphate kinase family, which was closer to thymidylate kinase than to cytosolic UMP-CMP kinase. Subcellular localization with green fluorescent protein fusion proteins illustrated that UMP-CMPK2 was localized in the mitochondria of HeLa cells and that the mitochondrial targeting signal was included in the N-terminal 22 amino acids. The enzyme was able to phosphorylate dUMP, dCMP, CMP, and UMP with ATP as phosphate donor, but the kinetic properties were different compared with the cytosolic UMP-CMPK. Its efficacy to convert dUMP was highest, followed by dCMP, whereas CMP and UMP were the poorest substrates. It also phosphorylated the monophosphate forms of the nucleoside analogs ddC, dFdC, araC, BVDU, and FuUrd, which suggests that UMP-CMPK2 may be involved in mtDNA depletion caused by long term treatment with ddC or other pyrimidine analogs. UMP-CMPK2 mRNA expression was exclusively detected in chronic myelogenous leukemia K-562 and lymphoblastic leukemia MOLT-4 among eight studied cancer cell lines. Particular high expression in leukemia cells, dominant expression in bone marrow, and tight correlation with macrophage activation and inflammatory response suggest that UMP-CMPK2 may have other functions in addition to the supply of substrates for mtDNA synthesis.

Nucleotide synthesis is a basic biological process for cell proliferation and almost all other physiological activities in the cell. Two pathways have been reported for nucleotide synthesis: the de novo pathway and the salvage pathway (1). In the de novo pathway, the synthesis of nucleotides starts from small molecules whereas in the salvage pathway, free nucleosides are directly used to synthesize ribonucleotides and deoxyribonucleotides.

Mitochondria only have the salvage pathway for nucleotide synthesis. To our knowledge, there are seven enzymes of this pathway that have been cloned and studied in human tissues: thymidine kinase 2 (TK2) (2–5), deoxynucleotidase 2 (mdN, or dNT2) (6–9), deoxyguanosine kinase (dGK) (10–13), adenylate kinase 2 (AK2) (14), adenylate kinase 3 (AK3) (15), adenylate kinase 3-like 1 (AK3L1, also known as AK4) (15, 16), and nucleoside diphosphate kinase NME4 (nm23-H4) (17, 18). Although the Drosophila melanogaster UMP-CMP kinase was reported to be a mitochondrial enzyme (19), there are no reports on a human mitochondrial UMP-CMP kinase or thymidylate kinase so far.

Antiretroviral or anticancer deoxynucleoside analogs can cause mtDNA depletion and lead to mitochondria dysfunction after long term treatment (20, 21). Mutations or deletion of either TK2 or dGK result in myopathic or hepatocellular forms of mtDNA depletion syndromes (MDS) (22–26). The MDS may also arise from deficiencies of other enzymes involved in mitochondrial nucleotide metabolism or transportation, such as the thymidine phosphorylase (TP) and the p53-controlled ribonucleotide reductase (p53R2) (27, 28). These proteins account for just a fraction of all MDS cases and defects in other genes may also be involved in the etiology of MDS.

To figure out the complete enzymatic steps of the salvage pathway for deoxynucleotide synthesis in mitochondria, we cloned and characterized the human homolog (hypothetical protein LOC129607, NCBI accession NP_997198) of a mouse gene, which was designated as thymidylate kinase family LPS-inducible member (TYKi) because of a putative thymidylate kinase domain (29). Previous studies showed that the expression of murine TYKi was induced or up-regulated by LPS and several cytokines (TNFα, IFNγ, IL-1β, IFNα) and was down-regulated by TGFβ (29–35), whereas there are no reports about its protein properties so far. Here, we cloned the full-length coding sequence of the human cDNA and found that the gene product was localized in the mitochondria of HeLa cells. We expressed this protein in insect cells and purified it to homogeneity. The recombinant protein phosphorylated CMP, UMP, dUMP, dCMP, dCDP, and dCDT, whereas dGMP, dTMP, dGDP, and dGTP were not phosphorylated.

2 The abbreviations used are: TK2, thymidine kinase 2; DTT, dithiothreitol; GFP, green fluorescent protein; mtDNA, mitochondrial DNA; ddC, 2’,3’-dideoxycytidine; dFdC, 2’,2’-difluorodeoxycytidine; araC, 1-β-D-arabinofuranosylcytosine; BVDU, 5-β-D-arabino-5-ido-2’-deoxyuridine; BVaraU, 1-β-D-arabinofuranosyl-5-(E)-2’-bromovinyl]uracil; FuUrd, 5-fluorodeoxyuridine; ddI, dideoxyinosine; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; IFNα, α-interferon; IFNγ, γ-interferon; IL-1β, interleukin-1β; TGFβ, transforming growth factor β; UMP-CMPK, UMP-CMP kinase.

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† The on-line version of this article (available at http://www.jbc.org) contains a supplemental table.

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Mitochondrial UMP-CMP Kinase

dCMP, dUMP, and monophosphates of the pyrimidine nucleoside analogs ddC, dFdC, araC, BVDU, and FdUrd. Based on its substrate recognition and intracellular location we designated this novel enzyme as mitochondrial UMP-CMP kinase and named it UMP-CMPK2. The relative phosphorylation efficacy of the natural substrates were dUMP > dCMP > CMP > UMP, which is different from the properties of cytosolic UMP-CMPK.

The characteristics and phylogenetic analysis demonstrated that this protein comes from a novel family of UMP-CMP kinases.

EXPERIMENTAL PROCEDURES

Cloning of Human UMP-CMPK2 cDNA—For PCR amplification of UMP-CMPK2 cDNA, the ATCC image clone 3063188 (GenBank™ ID: AW408129, BC089425) (ATCC: 9556333) was used as template, the forward primer containing the encoding sequence of the first 13 amino acids of hypothetic protein LOC129607 was 5'-CAC CAT GCC CTG CGC CCG CGG CCT GCT CCT GCG GCC ACT GTC G-3', the reverse primer was 5'-GAA GTA AAA TTA AGA TCG TCT TCC AGT TTT CTG-3'. PCR was performed in an Applied PCR201 Thermal Cycler (CLP Molecular Biology) with Platinum Pfx DNA polymerase (Invitrogen) and 2× PCRx Enhancer solution. The cycling parameters are 1× (94 °C, 2 min), 30× (94 °C, 30 s; 55 °C, 30 s; 68 °C, 2 min) and 1× (68 °C, 10 min). PCR products were cloned into pENTR™/SD/D-TOPO entry vector (Invitrogen) with TOPO cloning method to create an entry clone. The UMP-CMPK2 cDNA was constituted by recombination of this entry clone and pcDNA-DEST47 (Invitrogen) according to the manufacturer's instructions.

The HeLa cell line (American Type Culture Collection) was transfected with pN22-GFP and pUCMPK2-E448G-GFP according to the method described (19). The cells were stained with MitoTracker Red (Molecular Probes) 48 h after transfection. Cell fluorescence was imaged with a Nikon Eclipse E600 microscope equipped with a SPORT RT digital camera.

Expression and Purification of Recombinant UMP-CMPK2—The cDNA sequence without putative mitochondrial targeting signal was amplified by PCR with forward primer 5'-CAC CGG ATC CAT GGT CTG CGC TGG GCC CAT GG-3' (with BamH I site), reverse primer 5'-GAA TTA CTA GTG ATG GTG ATG GTG ATG CGG TTC ACT ATT CTG-3' (with EcoRI site and six histidine codons), Platinum Pfx DNA polymerase (Invitrogen) and 1× PCRx Enhancer solution. The cycling parameters are 1× (94 °C, 2 min), 30× (94 °C, 30 s; 58 °C, 30 s; 68 °C, 2 min) and 1× (68 °C, 10 min). PCR products were cloned into pENTR™/SD/D-TOPO to create an entry clone. The UMP-CMPK2Δ21-6His coding sequence from this entry clone was inserted into the BamHI-EcoRI site of the pBacPAK8 transfer vector (Clontech) to get a transfer construct. Recombinant virus was constructed by cotransfecting Spodoptera frugiperda (Sf9) cells with the transfer construct and BacPAK6 viral DNA according to the manufacturer's manual.

Protein expression in Sf9 cells and the affinity chromatography were modified from reported protocol (36). Sf9 cells were harvested 72 h after infection with 10 p.f.u. of recombinant virus. The cells were lysed, and the extract was cleared by centrifugation at 40,000 rpm × 30 min, 4 °C, using a Beckman 45TI rotor. The recombinant proteins were purified with TALON Metal Affinity Resin (Clontech), desalted on PD-10 column (Amersham Biosciences) and followed by anion-exchange chromatography with a MonoQ HR5/5 (Amersham Biosciences) column pre-equilibrated in low salt Buffer IEX (20 mM Tris-HCl, pH 8.5, 50 mM NaCl, 10% glycerol, 1 mM DTT, and 1× protease inhibitors). After elution in a linear gradient (10 ml) of Buffer IEX (0.05–1 M NaCl), the recombinant proteins were concentrated with Centricon 10 devices (Millipore) and applied to a Superose 6 10/300 GL column (Amersham Biosciences) pre-equilibrated in Buffer GF (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, and 1 mM DTT). The proteins were eluted at a flow rate of 0.30 ml/min with 1.5 bed volumes of Buffer GF. The calibration of column was carried out with Bio-Rad gel filtration standard.

Isoelectric Point Determination—The pI of recombinant protein was determined on a PhastSystem with IEF PhastGel IEF 3–9 media (Amersham Biosciences) according to the manufacturer's instruction.

Enzyme Assays—The nucleoside monophosphates and nucleoside analogs (Sigma) were added at a final concentration of 1 mM in a 10-μl reaction mixture containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM unlabeled ATP, 1 μCi of [γ-32P]ATP (3000 Ci/mmol) (Amersham Biosciences), and 0.1–1 μg of UMP-CMPK2Δ21-6His recombinant proteins. The reactions were carried out for 1 h (nucleoside monophosphates) or 2 h (nucleoside analogs) at 37 °C. 1 μl of reaction mixtures were
Mitochondrial UMP-CMP Kinase

Mitochondrial UMP-CMP kinase is currently recorded as hypothetical protein LOC129607 in Entrez Gene data base of NCBI. The genomic sequence is localized at 2p25.2. It is regarded as a thymidylate kinase family LPS-inducible member because its homology with mouse TYKi protein, which was LPS-inducible in macrophages and was supposed to have thymidylate kinase activity based on sequence similarity with thymidylate kinase (29). Although many ESTs already have been cloned, no mRNA or cDNA that covers the full-length coding region has been characterized.

To clone the full-length cDNA, we selected the ATCC cDNA clone IMAGE3063188 (NCBI accession BC089425) as our PCR template. By adding the coding sequence of 8 missing amino acids in the forward primer, we PCR amplified the full-length cDNA of UMP-CMPK2. Because this cDNA was derived from BC089425, which contains a point mutation at the codon of the 448th amino acid (GGA (codon of glycine) in BC089425 and GAA (codon of glutamic acid) in genomic DNA and all other ESTs), we designated the protein coded by this cDNA sequence as UMP-CMPK2-E448G. For protein expression, this point mutation was also corrected to glutamic acid (Glu, E) by using the genomic sequence. The PCR products were used as UMP-CMPK2-E448G-GFP fusion protein. As shown in Fig. 2, bottom panel, the green signal of UMP-CMPK2-E448G-GFP was also overlaid with the red signal of MitoTracker Red, which confirmed the mitochondrial localization of UMP-CMPK2 in HeLa cells.

Protein Expression and Purification of Recombinant UMP-CMPK2—At first, we thought that this protein would have TMPK activity based on BLAST results and interpretation of the mouse homolog (29). Initially, UMP-CMPK2 was expressed as His-tagged and GST-tagged recombinant proteins in Escherichia coli, respectively. We cleaved off the tags with protease and purified the proteins to high homogeneity (>95%). All enzymatic activity assays performed under conditions for TMPK activity only showed UMP-CMP kinase activity. No phosphorylation of dTMP was detected (data not shown). Considering a report that human TMPK activity was undetectable in transformed yeast or from proteins of several E. coli expression systems (38), we overexpressed His-tagged recombinant UMP-CMPK2 in insect cells and obtained pure proteins for enzymatic assay.

First we designed a new set of primers for PCR amplification. The forward primer started at the 22nd amino acid to remove the cleavable putative mitochondrial targeting signal. One ATG codon was added before the 22nd amino acid to start translation. The reverse primer was located around the position of stop codon, but the stop codon was replaced by a histidine codon followed by 5 more to encode the His6 tag. The E448G point mutation was also corrected to glutamic acid (Glu, E) by using the genomic sequence. The PCR products were used to construct recombinant Baculovirus carrying UMP-CMPK2Δ21-6His fusion gene.

The SF9 cells were infected with recombinant virus and harvested 3 days later. To characterize the properties of the enzyme, UMP-CMPK2Δ21-6His protein was first purified with affinity chromatography. The protein was quite pure according to the SDS-PAGE data (Fig. 3B, lane 2). To reach a higher purity we performed two more purification steps: anion-exchange and
Mitochondrial UMP-CMP Kinase

Expression of UMP-CMPK2 in Cancer Cells—A human Northern blot with poly(A) mRNA of eight different cancer cell lines was used. The mRNA expression was exclusively detected in chronic myelogenous leukemia K-562 and lymphoblastic leukemia MOLT-4 (Fig. 5). The former cell line had higher expression level than the latter one correlated to the actin mRNA abundance. Based on the RNA molecular standards, the estimated size of UMP-CMPK2 mRNA is about 3.3 kb, which is close to the size of NM_207315.2 (3009 bp) reported in NCBI. No expression was detected in other six cell lines.

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DISCUSSION

In mammalian cells, UMP-CMP kinase and thymidylate kinase are the only known pyrimidine nucleoside monophosphate kinases that have been identified. UMP-CMPK phosphorylates CMP, UMP, dCMP, and dUMP, whereas TMPK phosphorylates dTMP and dUMP (41). The majority of studies on mammalian nucleoside monophosphate kinases have been focused on cytosolic enzymes. Here, for the first time, we report a human mitochondrial UMP-CMP kinase, which was designated as UMP-CMP kinase 2.

The overlap of GFP signal and MitoTracker Red fluorescence indicated that UMP-CMPK2 was localized in the mitochondria of HeLa cells. The mitochondrial targeting signal was included in the first 22 amino acids, and the sequence around serine forms a sequence pattern similar to reported R-10 conserved sequence motif of mitochondrial targeting signal R\textsuperscript{X}F/I/LS\textsuperscript{X6} (42). The exact cleavage site needs to be determined by experimental methods.

The amino acid sequence of UMP-CMPK2 has high homology with TMPK. It contains putative motifs that are conserved used to phosphorylate cytidine analogs to get monophosphate substrates for UMP-CMPK2/Δ21-His. 1.0 μg of each enzyme per reaction was used; 2 h of incubation at 37 °C. C, human mitochondrial TK2 was used to phosphorylate uridine analogs to get monophosphate substrates for UMP-CMPK2/Δ21-His. 0.5 μg of each enzyme per reaction was used; 2 h of incubation at 37 °C.
Mitochondrial UMP-CMP Kinase

Mitochondrial UMP-CMP kinase has low affinity to pyrimidine ribonucleotides and it may mainly participate in dUTP (or just dUDP) and dCTP synthesis in mitochondria.

Deoxycytidine analogs and deoxuryridine analogs are important drugs widely used as antiviral and anticancer agents. For instance, ddC, BVDU, and BVarU are used as antiviral agents; dFdC, araC, and FdUrd are used in anticancer therapy (46–51). Using coupled enzymatic assays, recombinant UMP-CMPK2 could phosphorylate the monophosphate forms of all above analogs except BVarU. Under long term treatment, several deoxynucleoside analogs, such as ddC, can cause mtDNA depletion after incorporation into mtDNA by DNA polymerase γ (52). Our data propose the possibility that UMP-CMPK2 may be involved in the activation of ddC and other pyrimidine analogs in mitochondria. Further studies on UMP-CMPK2 and other enzymes of the same pathway, will contribute to increased knowledge that may reduce mitochondrial toxicity caused by these compounds.

The expression of UMP-CMPK2 was detected in two leukemia cell lines, and several sources have demonstrated a special expression pattern of this gene. The expression profile from UniGene data base (ID: Hs.7155) shows the highest expression level in bone marrow, whereas at all other tissues have relative rare transcripts. Many tissues, including tissues rich in mitochondria (such as muscle and heart), show no detectable transcripts of UMP-CMPK2. Among all investigated tumors, leukemia cells show the most abundant expression whereas other cells have very low levels or undetectable transcripts of this gene. Another expression profile from Genomics Institute of the Novartis Research Foundation also shows similar pattern. All these data suggest that UMP-CMPK2 may not be crucial for general cell activities, such as mtDNA synthesis, but play a role during hematopoiesis or lymphocytopenia.

Previous studies reported that only one UMP-CMP kinase was present in calf thymus, rat liver, or Yoshida sarcoma cells (41, 53), and there was no UMP-CMPK activity detectable in purified mitochondrial extracts from HeLa S3 cells (44) probably because of a very low expression level of UMP-CMPK2 in these tissues as shown by the expression profiles mentioned above. In addition, the antibodies developed for cytosolic UMP-CMPK may not recognize UMP-CMPK2 because of the relative long distance between them in evolution.

The studies of the mouse TYKi gene suggested a correlation between this protein and the immune response. LPS is a bacterial endotoxin which is a powerful activator of macrophages that in turn can recognize and destroy invading microorganisms and tumor cells and orchestrate the process of inflammation. Murine TYKi was reported to be significantly induced by LPS in macrophages, and this induction could be modulated by in TMPK, such as P-loop, LID domain, and catalytic site, as reported in mouse TYKi by Lee et al. (29). However, we were not able to detect any TMPK activity from highly purified recombinant UMP-CMPK2 expressed in insect cells or in E. coli. Because the mitochondrial targeting signal was removed in all expression systems used and all the protein preparations had similar UMP-CMP kinase activities, the N-terminal sequence is not likely to alter the enzyme activity. We conclude that the expressed recombinant protein is not a thymidylate kinase but instead a mitochondrial UMP-CMP kinase belonging to a novel protein family. This family is close to TMPK, but the distance to cytosolic UMP-CMPK is relatively far away, which supports the high phosphorylation efficacy of UMP-CMPK2 on dUMP.

Kinetic properties of UMP-CMPK2 were significantly different from those of cytosolic UMP-CMPK. Human cytosolic UMP-CMPK was reported to preferentially phosphorylate ribonucleotides (43, 44). In contrast, mitochondrial UMP-CMPK2 had higher efficacy for deoxyribonucleotides than for ribonucleotides. The best substrate was dUMP followed by dCMP, whereas the relative efficacy with CMP was lower than for dC. As the poorest substrate, UMP showed very low efficacy with the lowest Vmax and the highest Km. The Km values for CMP and UMP of the recombinant UMP-CMPK2 were higher than those of UMP-CMPK. The Km of dCMP is between the two reported values of UMP-CMPK. However, the Km for dUMP was lower than that of UMP-CMPK (43). All the specific activities for CMP and UMP were lower than those data of UMP-CMPK, but the Vmax/Km of dUMP from UMP-CMPK2 is higher (~1200–2400-fold) than the value of UMP-CMPK reported by Van Rompay et al. (43, 44). Referring to the regulation of UMP-CMPK by ATP and magnesium reported by Hsu et al. (45), we used 2 mM ATP and 6 mM Mg2+ in the kinetic assays for dCMP. Our observations showed that the kinase activity for dCMP of recombinant UMP-CMPK2 was significantly inhibited at high concentration of ATP (data not shown), which indicated that the activity of UMP-CMPK2 might be regulated by ATP concentration. In summary, current data indicate that mitochondrial UMP-CMP kinase has low affinity to pyrimidine ribonucleotides and it may mainly participate in dUTP (or just dUDP) and dCTP synthesis in mitochondria.

Deoxycytidine analogs and deoxuryridine analogs are important drugs widely used as antiviral and anticancer agents. For instance, ddC, BVDU, and BVarU are used as antiviral agents; dFdC, araC, and FdUrd are used in anticancer therapy (46–51). Using coupled enzymatic assays, recombinant UMP-CMPK2 could phosphorylate the monophosphate forms of all above analogs except BVarU. Under long term treatment, several deoxynucleoside analogs, such as ddC, can cause mtDNA depletion after incorporation into mtDNA by DNA polymerase γ (52). Our data propose the possibility that UMP-CMPK2 may be involved in the activation of ddC and other pyrimidine analogs in mitochondria. Further studies on UMP-CMPK2 and other enzymes of the same pathway, will contribute to increased knowledge that may reduce mitochondrial toxicity caused by these compounds.

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Mitochondrial UMP-CMP Kinase

myeloid differentiation protein-88 (29, 30). The number and size of mitochondria in macrophages were greatly increased after LPS induction (54). In other tissues, TYKi was also up-regulated by several inflammatory response-related cytokines (such as TNFα, IFNγ, IL-1β, and IFNα) and infections of parasites (31, 33, 55). All these expression profiles suggest that UMP-CMPK2 is actively involved in macrophage activation and the inflammatory response.

The human UMP-CMPK2 consists of 449 amino acids, which is about twice the size of UMP-CMPK. It can be separated into two domains according to sequence properties: the N-terminal domain and the C-terminal domain. All motifs participating in phosphoryl transfer are predicted to be located at the C-terminal domain, whereas there are no data available suggesting a function of the N-terminal domain. An interesting composition is that 9 of 13 cysteines crowd in the first 189 amino acid residues, which suggests that this region may have a tight or complex tertiary structure, oxidation/reduction activity or other special features. More than 60% of leucines, alanines, prolines, and glycines are located in the N-terminal domain. No leucine zipper pattern has been found and in contrast to the leucine-rich property, there are no isoleucines in this domain. Thus, the leucine rich domain may contribute with special properties, like protein-protein interactions, a possibility that will be further investigated. The two distinct domains of UMP-CMPK2: the N-terminal domain with unknown function and the C-terminal domain with the nucleoside monophosphate kinase function, suggest that UMP-CMPK2 may be a bifunctional protein with other biological functions in addition to its UMP-CMP kinase activity.

UMP-CMP kinase 2 should be responsible for phosphorylation of dCMP and dUMP in mitochondria (Fig. 6). Considering the pyrimidine metabolism pathway in cytosol, mitochondria may have their own thymidylate synthase. It is also possible that dCMP deaminase and dUTP diphosphatase activities exist in mitochondria. Although the newly discovered mitochondrial pyrimidine nucleotide carrier (PCN1) has high specificity for dTTP (56), it is not likely to provide sufficient dTTP for mtDNA synthesis in resting cells. The accumulated dTMP from phosphorylation of thymidine by TK2 (2–5), and from the import of cytosolic dTMP by a highly selective and active dTMP transport system (57), must be phosphorylated to dTDP and then dTTP through the salvage pathway. A thymidylate kinase should exist to catalyze this phosphorylation in mitochondria.

In summary, UMP-CMP kinase 2 is the first pyrimidine nucleoside monophosphate kinase that has been identified in human mitochondria. The expression profiles of UMP-CMP kinase 2 suggest that the dUDP metabolism pathway in mitochondria may play a unique role. Our current studies revealed its different characteristics compared with cytosolic UMP-CMP kinase. Further studies of this enzyme will contribute to further understanding the salvage pathway for nucleotide synthesis in mitochondria, to elucidate the actual role of the dUDP metabolism pathway and to reduce the mtDNA damage caused by nucleoside analogs.

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Mitochondrial UMP-CMP Kinase

JANUARY 18, 2008 • VOLUME 283 • NUMBER 3 • JOURNAL OF BIOLOGICAL CHEMISTRY

1571

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