Cloning and Characterization of a Family of Proteins Associated with Mpl*

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Thrombopoietin (TPO) controls the formation of megakaryocytes and platelets from hematopoietic stem cells via activation of the c-Mpl receptor and multiple downstream signal transduction pathways. We used two-hybrid screening to identify new proteins that interacted with the cytoplasmic domain of Mpl, and we found a new family of proteins designated A2D (for Ataxin-2 Domain protein). The A2D are 130-kDa proteins that have three regions similar to those of Ataxin-2, the gene product causing familial type 2 spinocerebellar ataxia. A2D has several isoforms with different C-terminal domains, all produced from a single gene by alternative splicing. Northern blotting indicated that the A2D gene is widely expressed in immortalized cell lines and hematopoietic and fetal tissues. A2D proteins were constitutively associated with Mpl in vivo in human hematopoietic UT7 cells. TPO also caused the release of A2D from the activated receptor, and the phosphorylation of A2D on tyrosines residues was dependent on the Mpl C-terminal domain. Finally, A2D bound to the unstimulated erythropoietin receptor, whereas erythropoietin caused dissociation from the erythropoietin receptor, suggesting that A2D proteins are new components of the cytokine signaling system.

TPO† is the primary physiological regulator of megakaryopoiesis (1). TPO acts via Mpl (2), a cytokine receptor originally identified as the cellular homologue of the product of the myeloproliferative leukemia virus (MPLV) oncogene (3). Mpl is found only in tissues that support hematopoiesis, the bone marrow, spleen, and the fetal liver; it is also abundant in the marrow, spleen, and the fetal liver; it is also abundant in the fetal liver; it is also abundant in the fetal liver; it is also abundant in

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‡ The abbreviations used are: TPO, thrombopoietin; EPO, erythropoietin; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; Jak, Janus kinase; SH, src homology; PTB, phosphotyrosine binding; EPO-R, erythropoietin receptor; IL, interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; TOB, transducer of erbb-2; ORF, open reading frame; MPLV, the myeloproliferative leukemia virus; WT, wild type.

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The gene encoding Ataxin-2, the product of the SCA2 locus, is defective in type 2 spinocerebellar ataxia (25–27), one of the familial neurodegenerative diseases characterized by unstable CAG repeats, expanded in patients (28, 29). These disorders result in progressive loss of neurons from the cerebellum, brain stem nuclei, and spino-cerebellar tracts. The only feature shared by the genes causing these diseases is that they have CAG repeats encoding polyglutamines. CAG repeat diseases all have such features as ubiquitination, aggregation of full-length or truncated proteins, and cleavage by caspase (30, 31). But just how the expansion of the repeat triggers neuron death remains unclear. Although SCA2 only affects the cerebellum and brain, Ataxin-2 transcripts are found in various tissues. The protein (145 kDa) is located in the cytoplasm and the trans-Golgi network. Several putative functional motifs have been identified in the Ataxin-2 protein (caspase3 cleavage site and clathrin-mediated trans-Golgi signal), but its biological function is unknown.

The present report describes the molecular cloning and characterization of several A2D 130-kDa proteins isoforms, each with different C-terminal domains. We show that the A2D proteins are most abundant in hematopoietic and fetal tissues. A2D proteins are constitutively associated with Mpl in vivo in hematopoietic cells and are released from the receptor upon its activation. TPO triggers the phosphorylation of A2D on tyrosine residues in a way that is dependent on the presence of the Mpl C-terminal domain. We also find that A2D proteins are constitutively associated with the endogenous erythropoietin receptor (EPO-R) and EPO is caused to dissociate from the receptor, suggesting that A2D proteins are new components of cytokine signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cytokines**—A-protein, leupeptin, phenylmethylsulfonyl fluoride, orthovanadate, octyl 3–1028 of A2D, deleting the C-terminal domains of the PEG-MGDF) was a generous gift from a Jurkat cell line cDNA library cloned into the NorI site of pSPORT plasmid, kindly provided by Dr. L. Maouche-Chreitien (INSERM U474). A total of 18 clones with inserts up to 4050 bp were obtained. The cDNAs contained the majority of the coding sequence. The remaining 5’ sequence was obtained from a human blood cDNA library cloned in λZAP ExpressTM/EcoRI vector (Stratagene number 938202) that allowed us to clone three complete cDNAs. Both strands of the isolated clones were sequenced on an Applied Biosystem automated DNA sequencer. The complete sequence of A2D-A cDNA corresponding to the longest cDNA can be found in GenBankTM under accession number AJ311790. The 3’ parts of the cDNAs were heterogeneous, generating isoforms with different C-terminal domains termed A2D-B, -C, -D, and -E (see “Results” and Fig. 4). One of these clones also contained an internal in-frame deletion from nucleotides 633 to 908 that deleted an internal ATG. The M37 clone isolated by two-hybrid screening extended from A2D-D nucleotide 2215 to the poly(A) tail.

**Cloning Genomic Sequences**—Genomic DNA surrounding position 261 in cDNAs was cloned by PCR with 1 μg of genomic DNA from UT7 and Jurkat cell lines with the following oligonucleotides: sense, 5’-GCAAACGCGCGCAGCGCGCGAGC-3’ (positions 390–408) with the Advantage-GC Genomic PCR kit (CLONTECH) under the following conditions: 1 cycle at 94 °C for 5 min; 5 cycles at 94 °C for 5 s, 68 °C for 2 min; 5 cycles at 94 °C for 5 s, 65 °C for 30 s, 68 °C for 2 min; 15 cycles at 94 °C for 5 s, 60 °C for 30 s, 68 °C for 2 min; and 1 cycle at 68 °C for 7 min. The concentration of GC-Melt was 1 μl. The PCR products were cloned in the pCR 2.1-TOPO vector with the PCR 2.1-TOPO cloning kit (Invitrogen), and the nucleotide sequence of 4 clones of each cell line was determined.

**Northern Blot Analysis**—A human multiple tissue Northern blot (CLONTECH) containing ~1 μg of poly(A)+ RNA from each tissue (hematopoietic, fetal, and adult) was hybridized according to the instructions of the manufacturer using a 1.5-kb EcoRI insert from the genomic A2D clone as probe. Northern blotting of human and murine immortalized cell lines was performed using 10 μg of total RNA from each cell line.

**Chromosomal Localization**—Human metaphase cells for fluorescence in situ hybridization were prepared from phytohemagglutinin-stimulated lymphocytes by using the thymidine synchronization and bromodeoxyuridine release technique for banding. The A2D cDNA probe was radiolabeled by the nick translation procedure (Invitrogen) and used for fluorescence in situ hybridization, which was performed as described previously (36).

**Antibodies**—Anti-A2D antibodies were obtained by cloning the cDNA coding for A2D amino acids 927–1057 in-frame into the expression vector pGex-4T2 (Amersham Biosciences). The resulting recombinant protein was purified by affinity chromatography with glutathione-Sepharose™ 4B beads and used to immunize rabbits. A monoclonal antibody M1 (Eastman Kodak Co.), specific for the FLAG epitope tag, was used to detect Mpl. Anti-phosphotyrosine monoclonal antibodies 4G10 and PY72 were provided by Dr. Drucker (Portland, OR) and antibodies 4G10 and PY72 were provided by Dr. Drucker (Portland, OR) and anti-A Jak2 antiserum was from Upstate Biotechnology, Inc. (catalog number 06-255). The anti-EPO-R antiserum used for immunoprecipitation was provided by Dr. Mayeux (INSERM U363, ICGM), and antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology, Inc. (sc-695).

**Cell Culture, Stimulation, and Transfections**—Growth factor-dependent human megakaryoblastic UT7 cells expressing murine FLAG-Mpl were cultured in α-minimum essential medium supplemented with 2 ml of calf serum and 0.5 mg of recombinant human colony-stimulating factor (GM-CSF) or 2 units/ml EPO. Before TPO stimulation, cells were washed twice in serum-free medium and suspended in α-minimum essential medium supplemented with 0.4% bovine serum albumin. Stainations were performed by adding either 100 ng per ml recombinant Hu-PEG-MGDF or 10 ng TPO mimetic peptide. For EPO stimulations, exponentially growing UT7 cells were washed.
binding of M37 to cytoplasmic deletion mutants of Mpl (Fig. 1). Removal of the 24 C-terminal amino acids of Mpl (Δ98–121) prevented the interaction with M37, whereas this 24-amino acid fragment alone interacted actively with M37. M37 also did not interact with Lex-Lamin or Lex-RasV12 showing that the M37/Mpl interaction was specific.

We assessed the expression of M37 transcripts by Northern blotting on various human hematopoietic tissues. Hybridization with the M37 probe revealed a set of transcripts with two major forms, 4 and 4.5 kb (see below and Fig. 4). M37 was also abundantly expressed in purified megakaryocytic CD41+ cells, whose proliferation and maturation are controlled by TPO.

M37 is an incomplete cDNA since it contained a 1.5-kb insert including a poly(A) tail. The M37 probe was used to screen cDNA libraries from human circulating blood cells and Jurkat cell line and to isolate full-length cDNA. A total of 21 independent clones were purified and analyzed. The largest was 4380 nucleotides long, very close to the size of the transcript observed by Northern analysis. DNA sequencing followed by sequence alignment with the FASTA program revealed a match with the cDNA of the human Ataxin-2-like protein A2LG (GenBankTM accession number AF034373).2 There were several discrepancies between our cDNAs and the A2LG sequence. (i) The 5’ 148 nucleotides of the A2LG published sequence were not present in our cDNAs. This sequence corresponds to repeated sequences in the human genome, as shown by GenBankTM data base analysis. (ii) 6 point mutations did not change the amino acid sequence, whereas 4 insertions of single nucleotides in our clones changed the reading frame in two regions of the cDNA as follows. 1) Insertion of a C at nucleotide 261 led to a translation initiation different from that of A2LG protein. Therefore, A2D and A2LG proteins have different N-terminal domains upstream of amino acid 32 of A2D. 2) Insertions of single nucleotides at positions 1454, 1465, and 1508 modified 18 amino acids of the A2LG published sequence. We cloned the corresponding exon from the genomic DNAs of UT7 and Jurkat cells (see “Experimental Procedures”) to verify the insertion at position 261. This insertion was found in the genomic clones and was also present in 5 overlapping cDNAs from a human circulating blood cell library taken from 4 adult males. A polymorphism at this position is therefore very unlikely. We confirmed our nucleotide sequence several times on both strands, and we designated this sequence as A2D-A for Ataxin-2 Domain Protein. This sequence has been registered in the GenBankTM data base under accession number AJ317970. We believe that A2LG and A2D-A are produced by transcription of the same gene.

The sequence of the A2D-A cDNA revealed an open reading frame extending from nucleotide 1 to 3396. The first methionine-encoding sequence at position 169 is preceded by a consensus sequence for initiating translation of a 1076-amino acid protein (Fig. 2A). Although the sequence upstream of nucleotide 169 has no stop codon, the entire encoded protein sequence is probably in A2D-A since in vitro transcription/translation generated a 130-kDa protein, very close to the size of the A2D protein in UT7 cell lysates (see below Fig. 5B). The A2D-A sequence also contained 2 additional in-frame ATGs (amino acids 183 and 315) flanked by nucleotides that fit the Kozak consensus that could be alternative translation initiation sites.

Our data indicate that the A2D-A protein is very rich in proline (15%) and serine (11%) and contains 27 tyrosines. The protein is basic except for a highly acidic region (residues 169–325). The sequence contains no obvious functional do-

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2 J. R. Xia, C. Liu, D. Wang, Q. Ruan, and H. Deng, unpublished results.
main, but it has three substantial regions similar to those of Ataxin-2. The first (amino acids 208–352) is 60% similar to Ataxin-2 (Fig. 3A) and is also conserved in a putative protein of Drosophila (GenBank™ accession number AE003708). The second (amino acids 651–668) is 100% similar to Ataxin-2 and homologous to a short region of the antiproliferative transducer of ErbB-2 (TOB1) protein (Fig. 3B). The last (amino acids 895–941) is 60% similar to Ataxin-2 (Fig. 3C). The N-terminal portion of A2D also contains 8 interrupted glutamines replacing the polyglutamine tract of Ataxin-2.

A heterogeneity at the 3′ end of the cloned cDNAs, compared with A2D-A, generated proteins with different C-terminal domains (Fig. 2B). They contained internal deletions affecting the reading frame as follows: 4 clones that lacked nucleotides 3308–3875 were designated as A2D-B (GenBank™ accession number AJ317971); one clone A2D-C lacked nucleotides 3308–3457 (GenBank™ accession number AJ317972); 8 clones A2D-D lacked nucleotides 3253–3851 (GenBank™ accession number AJ317973); and 4 clones A2D-E lacked nucleotides 3253–3874 (GenBank™ accession number AJ317974). The predicted amino acid sequences of the C-terminal domains of these isoforms are indicated in Fig. 2A. A cDNA library prepared from purified human megakaryocytes was similarly heterogeneous (data not shown). We derived a probe S (Fig. 2C), from nucleotides 3302–3840, which discriminated between these transcripts in Northern blots, revealing only the longest 4.5-kb transcript corresponding to A2D-A.

Mapping of the human gene by fluorescence in situ hybridization with the A2D cDNA probe revealed two loci on chromosome 16p11 and 7p21 (data not shown). By screening a human genomic library with the same probe, we cloned part of the A2D gene, including exons corresponding to cDNA nucleotides 1100–3700, and a pseudogene that had been located by others on chromosome 7p21 (GenBank™ accession number AC005014). We did not isolate any related gene, so we presume that the A2D gene is unique and located on chromosome 16. Moreover, sequence analyses indicated that the different transcripts A2D-A to -E were issued from this single gene by alternative splicing. In addition, the A2D and ATAXIN-2 genes

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3 E. Ryan and C. Wilson, unpublished results.
have a similar exon-intron structures (37), suggesting that they came from a common ancestor (data not shown).

**Tissue Distribution of A2D Transcripts**—As described above, the A2D mRNAs are found in hematopoietic tissues, with the highest expression in thymus, lymph nodes, and spleen. A2D transcripts are also abundant in fetal tissues, particularly in the fetal kidney (Fig. 4). The only adult organs that contains abundant A2D mRNAs is the testis. Transcripts were also weakly detected in the pancreas and placenta. The 4.5-kb isoform was the major transcript observed in fetal tissues, spleen, and lymph nodes, whereas the shorter form was predominantly detected in lymphocytes. A2D transcripts were also highly expressed in most immortalized human and murine hematopoietic cell lines; these also contained additional mRNAs ~3.7 kb long.

**A2D Proteins Associate with Mpl in Vivo**—The DNA sequence of the M37 clone corresponding to the 3’-half of the A2D cDNAs predicted two overlapping reading frames (ORF1 and ORF2), one of 347 codons and the other of 408 codons. We raised rabbit polyclonal antiserum against putative proteins from ORF1 in-frame with the Gal4 activation domain and ORF2 corresponding to the A2D reading frame to identify proteins encoded by this cDNA. These antibodies were tested both in vitro translation products and on UT7 lysates. They included several isoforms with different C-terminal domains.

As described above, the 130-kDa band probably includes sequences corresponding to the 3’-half of the A2D cDNAs. We used a coprecipitation approach in the UT7/Mpl cells that precipitate from UT7 lysates that was not precipitated by preimmune serum. This serum was designated preimmune serum. This serum was designated preimmune serum. This serum was designated preimmune serum. This serum was designated preimmune serum.

In agreement with these results, immunofluorescence anal-
ysis of UT7/Mpl cells with anti-A2D antibodies revealed staining at the cell periphery (data not shown). In addition, subcellular protein fractionation of UT7/Mpl cells (Fig. 7) indicated that A2D proteins were mainly expressed in the light membranes and to a lesser extend in the heavy membranes corresponding to endoplasmic reticulum/Golg fraction. Weak bands were also detected in the cytosolic and nuclear fractions. Fractions were identified by the presence of the caspase-3 in the cytosol and of Mpl in the light membranes.

**TPO Induces a Transient Tyrosine Phosphorylation of A2D**

That Is Dependent on the Mpl C-terminal Domain—Cytokines induce tyrosine phosphorylation of molecular targets involved in signal transduction. As A2D proteins are rich in tyrosine residues and associate with Mpl, we measured the capacity of TPO to stimulate the phosphorylation of A2D proteins. Starved UT7/Mpl cells were stimulated or not with TPO for various times, and their lysates were immunoprecipitated with anti-A2D or preimmune antibodies and then resolved by SDS-PAGE. Immunoblotting using anti-phosphotyrosine antibody (Fig. 8A) showed that a 130-kDa tyrosine-phosphorylated protein was specifically precipitated by anti-A2D antibodies after TPO stimulation. Stimulation also resulted in the detection of a nonspecific 110-kDa band by preimmune serum. The phosphorylated p130 in TPO-stimulated cells was detectable by 2 min and then decreased after 20 min. Reprobing with anti-A2D antibody demonstrated that this 130-kDa phosphorylated protein comigrated with A2D. We also detected other A2D-associated proteins whose tyrosines were phosphorylated; a 40-kDa protein (Fig. 8A), which has not yet been identified, was strongly and stably phosphorylated after TPO stimulation, and phosphorylated Mpl was also detected when the membranes were overexposed (Fig. 8B). The overall pattern of phosphorylation after TPO stimulation is shown on the left panel of Fig. 8A.

We confirmed that the phosphorylated 130-kDa protein corresponded to A2D, using UT7/Mpl cells expressing a Myc-tagged A2D protein lacking its N-terminal domain (amino acids 1–314 deleted). This protein has a molecular mass of 100 kDa that is easily distinguished from the endogenous 130-kDa phosphorylated protein. Cells were stimulated or not with TPO and phosphorylated A2D proteins were precipitated (IP) with anti-A2D antibody. Immunoblotting and whole cell lysates were analyzed by SDS-PAGE and anti-A2D immunoblotting. The membrane was reprobed with anti-EPO-R antibodies. Reciprocally (bottom line), cell lysates were immunoprecipitated with anti-A2D antibodies or preimmune serum (pi). Immunoprecipitated and whole lysates were analyzed by SDS-PAGE and immunoblotting (IB) with anti-EPO-R.
Fig. 7. Western blotting to detect A2D in subcellular protein fractions of UT7/Mpl cells. H, heavy membranes; L, light membranes; C, cytosolic fraction; N, nuclear fraction. Proteins (100 µg) were loaded in each lane and detected first with anti-A2D antiserum, then with anti-caspase3, and anti-FLAG antibody to detect Mpl. IB, immunoblot.

Fig. 8. A2D tyrosine phosphorylation induced by TPO. A, UT7/Mpl cells were incubated or not with TPO for different times at 37 °C and lysed in 30 mM octyl β-D-thioglucopyranoside. Lysates (2 × 10⁷ cell eq) were immunoprecipitated (IP) with preimmune serum (Pi) or anti-A2D antibodies and resolved on SDS-PAGE in parallel with cell lysates followed by anti-phosphotyrosine (PTyr) immunoblot (IB). The membrane was reprobed with anti-A2D antibodies. B, UT7/Mpl cell lysates (3 × 10⁷ cell eq) were immunoprecipitated in the same conditions as in A. Anti-phosphotyrosine immunoblot was overexposed to detect tyrosine-phosphorylated Mpl. The membrane was reprobed with anti-A2D antibodies.

Fig. 9. TPO-induced tyrosine phosphorylation of A2D-Myc (ANter). UT7/Mpl cells transfected with an N-terminal-truncated form of a Myc-tagged A2D expression vector were stimulated or not with TPO for 5 min and lysed. Lysates were immunoprecipitated (IP) with anti-Myc antibody, separated by SDS-PAGE, and immunoblotted (IB) with anti-phosphotyrosine (PTyr) antibodies. The membrane was reprobed with anti-Myc antibody.

expressing wild type Mpl (WT) (Fig. 10A). Cells expressing WT-Mpl and truncated Mpl-Δ34 were stimulated with TPO for 5 min, and their lysates were immunoprecipitated with anti-A2D antibodies and loaded on SDS-PAGE. Immunoblotting with anti-phosphotyrosine (Fig. 10B) indicated that A2D was phosphorylated in cells expressing WT but not in UT7/Mpl-Δ34 cells. Reprobing the membrane with anti-A2D antibodies showed that the same amounts of A2D protein were precipitated in both cell lines. Because the tyrosines of Mpl-Δ34 are not phosphorylated after TPO stimulation and because Mpl-Δ34 lacks the tyrosine residues 112 and 117, the major sites of receptor phosphorylation (17), receptor activation was verified by assessing Jak2 activation. Immunoprecipitation with anti-Jak2 antibodies, followed by immunoblotting with anti-phosphotyrosine antibody, demonstrated similar phosphorylation of Jak2 in both cell lines upon stimulation (Fig. 10C). Hence, Mpl-Δ34 is functional; thus, the need for the C-terminal part of Mpl cytoplasmic domain for A2D recruitment and tyrosine phosphorylation agrees with the two-hybrid results involving this region for A2D binding.

A2D Proteins Associate with EPO-R in Vivo—As A2D proteins are mainly present in the hematopoietic cells of many lineages, we determined whether they also interacted with another receptor belonging to the cytokine receptor family. We investigated the ability of A2D to bind to the EPO-R, as Mpl and EPO-R have structural and functional similarities. We used UT7 cells for their high surface EPO-R expression and their proliferation in response to EPO. Cells were starved for 16 h and then stimulated or not with EPO. The EPO-R was immunoprecipitated, and the immunoprecipitates were immunoblotted with anti-A2D antibodies. A2D proteins were found associated with the EPO-R in unstimulated UT7 cells (Fig. 6B). The amounts of A2D coprecipitated with the EPO-R decreased markedly after 10 min of EPO stimulation, whereas the amounts of receptor remained constant, as shown by reprobing the membrane with anti-EPO-R antibodies. Reciprocally, a 66-kDa protein that reacted with anti-EPO-R antibodies was coprecipitated with A2D from unstimulated UT7 cells, and adding EPO decreased the amounts of EPO-R bound to A2D proteins (Fig. 6B). This indicates that A2D is constitutively associated with EPO-R and dissociates after ligand stimulation, as is the case with Mpl.
New Mpl-associated Proteins

A2D binds to cytokine receptors Mpl and EPO-R. A2D is associated with these unoccupied receptors in vivo, and stimulation with TPO or EPO causes the rapid dissociation of A2D from the activated receptor. A2D proteins also contain proline-rich regions with several potential target sequence for MAPK phosphorylation. How- ever, they also associate with the EPO-R, dissociate from the activated receptor, and are tyrosine-phosphorylated by EPO (data not shown). Finally, A2D proteins may also function as scaffold proteins. If so, their overproduction would sequester downstream signaling molecules involved in proliferation. Indeed, the sequence of A2D proteins contains two tandem repeats (between amino acids 677 and 693) of consensus sequences ((R/K)S PP) for binding to 14-3-3 proteins that interact with many key signaling molecules and regulate signal transduction events (50). A2D proteins also contain proline-rich regions with several PXXP motifs that could bind to SH3 domains. The tyrosine residue 349 of A2D in the sequence YXXX corresponds to the consensus sequence for binding to the SH2-SH2 domain. A2D proteins are also rich in serine residues, some of them in a potential target sequence for MAPK phosphorylation. However, our preliminary experiments have shown no association between A2D and Shc, MAPK, or 14-3-3 by coimmunoprecipitation or pull-down experiments. Likewise A2D had no effect on MAPK activation (data not shown). We still need to determine whether A2D proteins are adaptor molecules or initiate new signaling pathways.

In conclusion, we have identified a new family of proteins that are probably involved in TPO signaling. A single cell line produces proteins that have different C-terminal domains or lack their N-terminal region (p120 and p100) and are encoded by a single gene. A2D proteins are abundant in all hematopoietic lineages, indicating that they may be involved in other cytokine signaling systems. They also associate with the EPO-R, dissociate from the activated receptor, and are tyrosine-phosphorylated by EPO (data not shown). Finally, A2D proteins may be encoded by a gene belonging to a larger family because we detected a 160-kDa protein having epitopes similar to A2D proteins. The synthesis of this protein is greatly increased during megakaryocytic differentiation.4

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