Cloning and Characterization of a Gene Expressed during Terminal Differentiation That Encodes a Novel Inhibitor of Growth*

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We report here the cloning and initial characterization of a novel growth-related gene (EEG-1) that is located on the short arm of chromosome 12. Two spliced transcripts were cloned from human bone marrow and human erythroid progenitor cells: EEG-1L containing a 4350-nucleotide open reading frame encoding a putative protein of 1077 amino acids including a C1q-like globular domain, and an alternatively spliced transcript lacking exon 5 (EEG-1S) encodes a significantly smaller coding region and no C1q-like domain. Quantitative PCR revealed expression of both EEG-1 transcripts in all analyzed tissues. Plasmids encoding green fluorescent protein-tagged genes (GFP-EEG-1) were transfected into Chinese hamster ovary cells for localization and functional assays. In contrast to the diffuse cellular localization of the GFP control, GFP-EEG-1L was detected throughout the cytoplasm and excluded from the nucleus, and GFP-EEG-1S co-localized with aggregated mitochondria. Transfection of both isoforms was associated with significantly increased levels of apoptosis. Stable transfection assays additionally demonstrated decreased growth in those cells expressing EEG-1 at higher levels. Quantitative PCR analyses of mRNA obtained from differentiating erythroid cells from blood donors were performed to determine the transcriptional pattern of EEG-1 during erythropoiesis. EEG-1 expression was highly regulated with increased expression at the stage of differentiation associated with the onset of global nuclear condensation and reduced cell proliferation. We propose that the regulated expression of EEG-1 is involved in the orchestrated regulation of growth that occurs as erythroblasts shift from a highly proliferative state toward their terminal phase of differentiation.

Controlled growth of erythroid tissues represents a precisely regulated balance of proliferation and differentiation that can be examined in vivo and in vitro. Developmentally, relatively quiescent stem cells become committed erythroid progenitors in the bone marrow of adults, and those rapidly proliferating progenitors subsequently undergo terminal differentiation into circulating erythrocytes. The processes of proliferation, differentiation, and apoptosis are balanced to produce enough mature erythrocytes to satisfy the oxygen demands of the host. Acute and chronic tissue hypoxia results in additional erythroid stress responses (1). The erythroid lineage is one of the most prolific developmental pathways of adult human life with \( \sim 10^{11} \) mature erythrocytes produced weekly. In addition to normal erythropoiesis, dysregulation of those mechanisms that control erythroid growth may result in anemia, polycythemia, or erythroleukemia in humans (2). Hence, the well defined relationship between hypoxia and erythroid cell production as well as the association between dysregulated erythroid growth and disease make examination of erythroid growth and differentiation both interesting and relevant.

Erythropoiesis is primarily regulated by the hormone erythropoietin released from the kidneys in response to hypoxia. Erythropoietin is thought to permit or promote the survival of erythroid progenitors, and erythroblasts undergo apoptosis in its absence (3). In addition to erythropoietin, stem cell factor may be involved in a proliferation response to acute erythroid stress (4). At the opposite end of the spectrum, recovery from erythroid stress may be modulated by paracrine mechanisms involving the Fas ligand (5). Hence, several environmental signal transduction cascades exist to regulate the absolute number of circulating erythrocytes generated by each progenitor cell. The switch from committed progenitor cell proliferation toward terminal erythroid differentiation also depends upon regulation of cell cycle kinases and their inhibitors (6). Erythroid proliferation and differentiation are controlled globally by expression of erythroid-specific genes as well as more generic factors. Indeed, gene disruption studies have demonstrated that expression of specific transcription factors like GATA-1 (7), as well as those involved in more general developmental regulators like rbtn2, are required for effective erythropoiesis to occur (8).

In addition to the expression of several genes already known to be involved in the control of erythroid proliferation, differentiation, and apoptosis, we hypothesized that novel growth-regulating genes may be identified by gene profiling of developing erythroid cells. We described previously (9) a large number of growth-related genes that are up-regulated in response to the hormone erythropoietin. Those gene profiles also include numerous genes known to be generally involved in tissue differentiation and apoptosis. Here we report the identification, cloning, and initial characterization of a novel growth-regulating gene called EEG-1 expressed during terminal erythroid maturation.

MATERIALS AND METHODS

EEG-1 cDNA Cloning—Informatic analysis of transcription profiles of human erythroid cells (hembase.niddk.nih.gov/) was used to identify the gene studied here. Experimentally, forward 5′-TCTGTTAAACTTGGAGCTGAGAA-3′ and reverse 5′-AGTAGCTGAAGGCGGTTGAAGG-3′ primers were used to amplify template cDNA from a human bone marrow library by PCR amplification in a GeneAmp PCR System 9700 (PerkinElmer Life Sciences) with the following conditions: one cycle at 94 °C for 1 min; 30 cycles at 60 °C for 30 s, 72 °C for 3 min, and 94 °C
for 30 s; and 72 °C for 10 min. The amplified products were subcloned into the pCR®-TOPO® vector (Invitrogen) and sequenced. Amplification of cDNA 3'- and 5'-ends was then performed using a human bone marrow cDNA library to define the transcriptional boundaries of the gene. Full-length cDNA was then amplified by reverse transcription (RT)-PCR with cDNA prepared from human erythroid progenitor cells, using the forward primer derived from 5'-end cDNA sequence and reverse primer derived from 5'-end sequence. The PCR product was purified on an agarose gel and subcloned into pCR®-TOPO® vector (Invitrogen). The nucleotide sequences were determined using an ABI prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an ABI Prism 3100 genetic analyzer. Chromosomal location, intron-exon boundaries, and homology scores were assigned by comparing the cDNA sequence with those deposited in GenBank™ using BLAST programs (www.ncbi.nlm.nih.gov/).

**RT-PCR Analyses—** To assess the relative expression level of EEG-1L and EEG-1S transcripts in human tissues, the multiple tissue cDNA panel I (K1420-1) and human immune system MCTC™ panel (K1426-1) from Clontech were used as templates of real time PCR. Quantitative real time PCR was performed by an ABI Prism 7700 sequence detection system (PerkinElmer Life Sciences, Applied Biosystems) using gene-specific forward and reverse primers and dual-labeled fluorogenic internal probe. Dual-fluorescent nonextendable probes labeled with 6-carboxyfluorescein (FAM) at the 5'-end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end were used for the detection. The primers and probes for EEG-1L (forward 5'-CTGACTTCATCACTCTTCTGACTGACTTTCAATCTTG-3' and reverse 5'-CGTCTCATACACCACTCTCTCCACTA-3') were chosen for the primer sequences of a systematic effort to identify and characterize novel genes expressed during erythropoiesis, we identified two erythroid expressed sequence tags (EST). As shown in Fig. 1, those EST (AX57C03 and CL39A09) are homologous to regions in close proximity on chromosome 12, so we speculated that they might encode the same gene. Cloned and sequenced cDNA fragments from bone marrow and erythroid cell libraries were used to generate a 4.3-kb full-length cDNA fragment. Sequencing analysis demonstrated the presence of two EEG-1 cDNAs, which we term EEG-1L and EEG-1S.

**Identification and Cloning of Peak Expressions—** As part of a systematic effort to identify and characterize novel genes expressed during erythropoiesis, we identified two erythroid expressed sequence tags (EST). As shown in Fig. 1, those EST (AX57C03 and CL39A09) are homologous to regions in close proximity on chromosome 12, so we speculated that they might encode the same gene. Cloned and sequenced cDNA fragments from bone marrow and erythroid cell libraries were used to generate a 4.3-kb full-length cDNA fragment. Sequencing analysis demonstrated the presence of two EEG-1 cDNAs, which we term EEG-1L and EEG-1S.

**Flow Cytometry and Confocal Microscopy—** Flow cytometric analyses were performed using an EPICS ELITE ESP flow cytometer (Beckman Coulter), and 10,000 cells were analyzed from each sample. Experiments were performed in triplicate with similar results. To determine the expression level of the EEG-1L or EEG-1S, the cultured monolayers were washed and trypsinized to generate cell suspensions. GFP expression was quantitated by calculating the percentage of cells with negative, low, medium, and bright GFP intensities. GFP-expressing populations were identified from 5'-end fluorescence at levels greater than 2 S.D. above the control population. Fluorescence microscopy was also performed after transfection for comparison.

A Zeiss LSM 410 confocal microscope equipped with an external krypton/argon laser was used for localization studies. Image processing was performed using IPLabs (Scanalytics) and Adobe Photoshop 5.0. MitoTracker staining was done by incubation with 50 nm MitoTracker Red CMXRos (Molecular Probes) according to the manufacturer's protocol.

**Detection of Apoptosis—** Apoptosis studies were performed using two methods. First, dual-color flow cytometry was used to quantify annexin V-FITC staining of GFP-expressing cells 24 h after transfection using an Annexin V-PE Detection Kit (BD Pharmingen). For confirmation, TUNEL staining was performed with an In situ Cell Death Detection Kit, TMR Red (Roche Applied Science). The percentages of apoptotic cells among the transfected population were calculated by flow cytometry analyses of ≥5000 transfected cells (annexin V-PE) versus microscopic analyses of >100 cells (TUNEL) in triplicate experiments. All statistical analyses were performed by Student's t test.

**RESULTS**

**Identification and Cloning of Human EEG-1 cDNA—** As part of a systematic effort to identify and characterize novel genes expressed during erythropoiesis, we identified two erythroid expressed sequence tags (EST). As shown in Fig. 1, those EST (AX57C03 and CL39A09) are homologous to regions in close proximity on chromosome 12, so we speculated that they might encode the same gene. Cloned and sequenced cDNA fragments from bone marrow and erythroid cell libraries were used to generate a 4.3-kb full-length cDNA fragment. Sequencing analysis demonstrated the presence of two EEG-1 cDNAs, which we term EEG-1L and EEG-1S.

EEG-1L and EEG-1S were encoded by a 45-kb region of chromosome 12p11.21. Comparison of cDNA and genomic sequences suggested that human EEG-1 gene consists of at least 18 exons (Fig. 1B). All of the intron-exon junctions exhibit consensus sequences of eukaryotic splice junctions GT/AG rule (Table 1). A comparison of the EEG-1L and EEG-1S revealed that they were generated as splice variants of the same gene. EEG-1L lacks the 147-bp fragment corresponding to exon 13, whereas EEG-1S lacks the 83-bp fragment corresponding to exon 5 (Fig. 1C). Beginning with an ATG initiation codon located in exon 1, EEG-1L contains an open reading frame encoding a protein of 1077 amino acids. In contrast, the loss of exon 5 in EEG-1S results in a shifted reading frame that introduces a stop codon (TGA) within exon 6. The predicted protein is truncated by 279 amino acids in its C-terminal protein (Fig. 1C). A Kozak consensus sequence is present at the designated ATG, and the putative polyadenylation signal sequence AATAAA is found 26 bp upstream of the poly(A)-tail. No larger cDNA clones were generated by further attempts to amplify the cDNA 3'- and 5'-ends. Comparisons of an EEG-1 cDNA sequence to the GenBank™ database using the BLAST program revealed significant homology at protein or nucleotide levels among all the organisms examined except Caenorhabditis elegans. The EEG-1 cDNA sequence was submitted to the GenBank™ with accession number AF074491. Analysis of protein structure with the PROSITE data base revealed that the EEG-1L contains a C-terminal gC1q-like domain with significant homology to the gC1q-like domain of a number of proteins (10). Other predicted motifs include several putative post-translational phosphorylation motifs, one tyrosine-kinase phosphorylation site, one cAMP- and cGMP-dependent protein kinase phosphorylation site, several casein kinase II and pro-
tein kinase C phosphorylation sites, and the N-terminal nuclear localization sequence.

Tissue Distribution of EEG-1—RT-PCR was performed to determine the tissue distribution of EEG-1 isoforms due to low level signals detected by Northern analysis. Quantitative PCR and an RT-PCR assay with nested primers in BM1 and BM3 containing the complete open reading frame. B, genomic organization of the putative human erythroid expression gene. Exons are shown as boxes, the black areas of the boxes represent coding sequences, and the open box areas denote the 5'- and 3'-untranslated sequences. Introns are shown as black horizontal lines. The gene is drawn to scale, except intron II is truncated as indicated by //. C, schematic illustration of human EEG-1 splice variants. The alternatively spliced EEG-1 mRNA isoforms and the two corresponding EEG-1 proteins are schematically represented by solid boxes. The locations of ATG, stop codons TGA, and poly(A) signals are shown by vertical arrows. The EEG-1L cDNA does not contain the exon 15 sequence. Alternatively, EEG-1S does not contain exon 5, and the resulting EEG-1S reading frame is shifted to terminate in exon 6. The resulting EEG-1S open reading frame is thereby truncated and does not contain a C1q domain.

Intracellular Localization and Functional Analysis of EEG-1—To gain insight into the subcellular localization and function of the two EEG-1 isoforms, the EEG-1L and EEG-1S transcripts were fused to the C terminus of a pEGFP expression vector plasmid, and the fusion constructs were transfected into CHO cells. The cellular localization of the GFP-tagged proteins seen in a majority of cells (experiments in triplicate) are shown by the representative images in Fig. 3. Expression of the control GFP vector demonstrated the expected distribution in both the cytoplasm and nucleus. Transfection of the GFP-tagged EEG-1L protein exhibited a diffuse pattern throughout the cytoplasm, but exclusion from the nucleus was noted. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Where low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct. The pattern of GFP-tagged EEG-1S fluorescence was also distinct. Whereas low levels of GFP-EEG-1S were detectable in the cytoplasm, more intense fluorescence was seen in the perinuclear regions of those cells. Co-staining with a mitochondria-localized dye (MitoTracker) demonstrated that the perinuclear GFP-EEG-1S fluorescence was also distinct.
expression and cell growth. After that period, the culture plates were washed and examined by fluorescent microscopy. Unlike the clear GFP signals detected among the control GFP colonies after 2 weeks, dim or absent fluorescence was observed by microscopy in EEG-1L- or EEG-1S-transfected colonies. The colonies were also examined by flow cytometry to quantitate differences in the level of transgene expression. As shown in Fig. 4, expression of the control and tagged constructs was present at all levels when measured 24 h after transfection. After 2 weeks, almost no cells demonstrated high level expression in the EEG-1L or EEG-1S. This negative correlation between EEG-1 expression and growth was also present among populations expressing GFP at medium signal intensities in repeated experiments. As shown in Fig. 4, 17% of the control cells, compared with only 2% of tagged EEG-1L-transfected pools and 0% of the EEG-1S-transfected pools, possessed medium level fluorescence. These data demonstrate a growth disadvantage associated with the expression of tagged EEG-1L and EEG-1S.

The loss of high level EEG-1 expression in Fig. 4 led us to explore further the possibility that EEG-1 gene expression may be involved in the regulation of cell growth or death. Thus, we examined the percentage of apoptotic cells in those transfected
populations compared with the control vector (Fig. 5). Two methods (annexin-V binding and TUNEL) were used to analyze the apoptotic features of the transfected populations. Annexin V binds to apoptotic cells due to exposure of phosphatidylserine translocation from the inner to the outer layer of the plasma membrane at an early stage of apoptosis (11). At 24 h post-transfection, the percentage of annexin V-positive cells was significantly increased in EEG-1-transfected cells compared with cells transfected with the empty vector control (GFP control, 20 ± 9%; GFP-EEG-1L 51 ± 7%, *p < 0.05; GFP-EEG-1S, 59 ± 2%, *p < 0.05). For confirmation, we also measured apoptotic cell death 24–72 h after transfection by TUNEL assay. This assay detects cellular endonuclease-mediated ordered DNA fragmentation, a later event in apoptotic cell death (11). As shown in Fig. 5B, the TUNEL results confirmed the association between EEG-1 expression and apoptosis. A significant increase in TUNEL-positive cells was observed over the 72 h after transfection with GFP-tagged EEG-1L or EEG-1S. The percentage of TUNEL-positive cells did not significantly change after transfection of the control GFP plasmid.

Since cell growth appears to be inhibited by EEG-1 gene expression, we next determined the relative level of EEG-1 gene expression during erythropoiesis. For this, CD34+ cells from normal blood donors were cultured for 14 days for semisynchronous development of mature erythroblasts over that period (12). To determine the expression pattern of EEG-1 during erythroid differentiation of CD34+ cells, quantitative RT-PCR was performed with mRNA from cells collected every 48 h over the 2-week culture period. As shown in Fig. 6, EEG-1 expression is highly regulated during erythroid development. Both isoforms were present at low levels during the initial culture period as the CD34+ population underwent rapid proliferation. The transcription levels of both EEG-1 isoforms then rapidly increased and reach the maximal level on day 8 (984 ± 96, *p = 0.001). After day 10, their expression was down-regulated. On all days, the EEG-1S isoform was detected at higher levels. Combined with the low level of expression detected in unfractionated bone marrow, this highly regulated pattern suggests EEG-1 exerts its growth-related effects among cellular subsets rather than throughout hematopoietic development. In this culture system, days 7–10 represent a distinct erythroid transition from a highly proliferative phase toward terminal erythroid maturation marked by a loss of cell proliferation, nuclear condensation, shrinkage of cell size, and high level expression of hemoglobin associated with terminal erythroid maturation (12). Fig. 6 also demonstrates a similar pattern of regulated expression during erythropoiesis for other growth regulatory genes including those associated with cell cycle regulation and the anti-apoptosis gene BCL-2. The EEG-1 expression pattern most closely matched those genes involved with...
the regulation of the cell cycle (P21 and CDK2). Similar to EEG-1, the expression of the cyclin-dependent kinase inhibitor P21 gene reached peak levels on days 8–10. Increases were detected in the gene expression level of the CDK2 transcript slightly earlier during the culture period. Interestingly, increased levels of the anti-apoptotic BCL-2 mRNA occurred earlier during the 1st week of the culture period. No significant changes were observed in control G3PDH transcript.

**DISCUSSION**

Erythropoiesis entails a precisely controlled developmental process that integrates the growth-related themes of cell proliferation, differentiation, and apoptosis with erythroid-specific gene expression. Hence, we predicted previously that gene expression profiles from highly purified and developmentally staged erythroid cells provide a robust source of information regarding elements involved in the regulation and control of erythroid cell growth. In this study, we identified a previously uncharacterized growth-related gene that we call EEG-1. The gene is expressed from a 45-kb locus on human chromosome 12p11.21, and two EEG-1 splicing variants present in the erythroid lineage were studied. Informatic analysis of the EEG-1 open reading frame identified this gene as a new member of a family of genes containing a C1q globular domain (10). The family of genes containing a C1q globular domain includes the complement protein C1q itself (13), proteins associated with animal hibernation (14), and precerebellin (15). However, experimental evidence is required to determine whether this and other genetic motifs in the EEG-1 transcripts are functional in the translated protein. Of note, a cellular receptor for C1q globular proteins is located in mitochondria and elsewhere in cells (16).

In order to learn more about distribution and possible function of EEG-1, we transfected plasmids encoding GFP fused to the EEG-1L and EEG-1S isoforms into CHO cells. Transient transfections with plasmids expressing the proteins fused to green fluorescent markers, the cellular localization of the tagged EEG-1L and EEG-1S proteins were visualized. Unlike the control transfections with GFP alone, neither EEG-1L open reading frame identified this gene as a new member of a family of genes containing a C1q globular domain (10). The family of genes containing a C1q globular domain includes the complement protein C1q itself (13), proteins associated with animal hibernation (14), and precerebellin (15). However, experimental evidence is required to determine whether this and other genetic motifs in the EEG-1 transcripts are functional in the translated protein. Of note, a cellular receptor for C1q globular proteins is located in mitochondria and elsewhere in cells (16).

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