Translocon-associated protein α transcripts are induced by granulocyte-macrophage colony-stimulating factor and exhibit complex alternative polyadenylation

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Abstract The cloning of full length cDNA for the translocon-associated protein α subunit, previously called signal sequence receptor α, is reported as a result of differential display experiments in search of genes induced by granulocyte-macrophage colony-stimulating factor. Its messenger RNA was more abundant in growing cells than in either factor-deprived cells or quiescent cells and comprised four species, each having micro-heterogeneity, as a result of complex alternative polyadenylation apparently dependent on arrays of non-canonical polyadenylation signals. Radiation hybrid mapping of the gene showed that the gene is on the short arm of chromosome 6.

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Key words: Translocon; Signal sequence; Decorin; Granulocyte-macrophage colony-stimulating factor; Schizophrenia

1. Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine produced by a variety of cell types in response to specific stimuli. It stimulates the proliferation of myeloid and endothelial cells and regulates the functions of terminally differentiated myeloid cells (reviewed by Gasson [1]). When bound to the ligand, the cellular receptor for GM-CSF transduces a signal into the cell through a number of pathways, e.g. the JAK2 tyrosine kinase mitogen-activated protein kinase pathway [2–6], the JAK2 signal transducer and activator of transcription five proteins pathway and the phosphatidylinositol 3-phosphate kinase pathway. In addition, a number of other kinases are known to be phosphorylated on GM-CSF stimulation [7–10].

GM-CSF stimulation leads to the induction of transcription of a number of genes including c-fos, c-jun, junB [11,12], c-myc [13], egr-1 [14], c-fms [15] and lipoxygenase [16]. To identify more genes that are induced by GM-CSF and thereby facilitate our understanding of the GM-CSF function, we performed the differential display technique, which was recently developed to clone genes expressed in one population of cells but not in the other [17]. We found that translocon-associated protein (TRAP) α is induced in a growth factor dependent human myeloid cell line, TF-1, after stimulation with GM-CSF. We also found that TRAP α messenger RNA (mRNA) undergoes complex alternative polyadenylation. Identification of a membrane protein residing in the endoplasmic reticulum (ER) as a GM-CSF-induced gene might represent a so far unvisited aspect of the GM-CSF function.

2. Materials and methods

2.1. Cells

The TF-1 cell line [18] and MG-63 osteosarcoma cell line [19] were purchased from the American Type Culture Collection (Rockville, MD, USA). TF-1 cells were cultured in medium supplemented with 25 ng/ml recombinant human GM-CSF (Immunex, Seattle, WA, USA).

2.2. Differential display

Differential display was done principally by the method of Liang et al. [17,20]. Northern blot hybridization was performed according to the standard protocol [21]. Even loading of samples between the lanes was verified by staining the blot with methylene blue.

2.3. Nucleotide sequence analyses

The nucleotide sequence was analyzed on both strands of the cloned cDNAs utilizing either the Core Facility of UCLA or an ABI Prism 377 sequencer (Perkin-Elmer, Norwalk, CT, USA). Either the Dye Terminator Cycle Sequencing Ready Reaction kit or Dye Primer Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Norwalk, CT, USA) were used for the reaction.

2.4. Analyses of TRAP α cDNA

Using the primers designed from the sequence of the cloned, differentially displayed fragment, rapid amplification of cDNA ends (RACE) reactions were done in each direction with the Marathon cDNA amplification kit (Clontech Lab., Palo Alto, CA, USA). Amplified cDNA bands were cloned into a plasmid vector and sequenced from their adaptor end, enabling us to design primers to amplify full length cDNA. The full length cDNA was then amplified, cloned into the vector and sequenced. To analyze TRAP α transcripts, a 3′-RACE reaction was performed using the same kit, starting from a primer that was designed to anneal to the 5′-untranslated region of the TRAP α cDNA. PCR fragments were then cloned and sequenced. The nucleotide sequence of the full length cDNA has been deposited in the GenBank database under accession number AF156965.

2.5. Flow cytometric analyses of the DNA content

Cells were harvested, rinsed with PBS and fixed with 66% methanol in PBS. The cell cycle distribution was measured by incubating cells in DNA staining solution (15 μg/ml propidium iodide in PBS). Fluorescence was excited at 488 nm and measured at 575 nm using a FACSCAN flow cytometer (Becton-Dickinson, San Jose, CA, USA). The percentages of cells within the G1, S and G2/M phases of the cell cycle were determined by analysis with the software provided by Becton-Dickinson.

2.6. Radiation hybrid mapping

Radiation hybrid mapping was done with the GeneBridge 4 RH panel purchased from Research Genetics (Huntsville, AL, USA). The PCR products from the gene panel were analyzed on an agarose gel. Results of two separate reactions were submitted to WICGR Mapping Service via internet (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl), which gave us the computed result.

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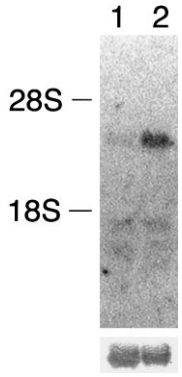


Fig. 1. Northern blot analyses of TF-1 RNA. 10 μg of total cellular RNA from factor-deprived TF-1 cells (lane 1) and TF-1 cells growing in GM-CSF-supplemented medium (lane 2) were separated in a 1% formaldehyde gel and transferred to a nylon membrane. DNA eluted from a differentially expressed band was labelled and hybridized to the membrane. The migration pattern of 28S and 18S ribosomal RNA is shown on the left. The staining of the 28S ribosomal RNA on the blot is shown in the lower panel.

3. Results

3.1. Differential display

To isolate transcripts induced by GM-CSF, we set out to analyze mRNA expression by the differential display technique. From the TF-1 cell line, which was initially established from an erythroleukemic patient and exhibits multiple factor dependence, we isolated RNA before and after 60 h deprivation of GM-CSF. Using the differential display technique, we identified a band specific to RNA from cells supplemented with the factor. When used as a probe for a Northern blot analysis, the DNA recovered from the band detected a 3.3 kb band of which the expression was upregulated by GM-CSF (Fig. 1). The DNA was cloned into a plasmid vector (called R60 hereafter). Nucleotide sequence analysis of R60 and subsequent searching of the GenBank revealed its identity with one of two 3′-ends of reported decorin cDNA [22]. However, this may not be an authentic cDNA end of decorin, since Danielson et al. had noted that this cDNA end did not detect bands matching the size of decorin mRNA in a Northern blot analysis [23]. This fact prompted us to look further into the R60 sequence.

3.2. Cloning of the full length cDNA

To obtain more sequence information on the transcript encompassing R60, we performed RACE reactions. Successfully amplified and cloned, the full length cDNA had a predicted open reading frame (ORF) encoding the previously reported TRAP α at its 5′-end and included the R60 sequence at its 3′-end (Fig. 2).

3.3. Expression pattern of TRAP α RNA

To determine if the cDNA that we cloned is expressed in cells, we probed Northern blots with a 1.5 kbp fragment encompassing the whole coding region of the TRAP α cDNA. As expected, the probe detected a 3.3 kb band which was of the same size as the band detected with R60 and with the size of the cDNA itself (Fig. 3b), suggesting that the cloned cDNA roughly represented a full length fragment. Remarkably, the probe also detected several bands of smaller sizes (see below). Next, the expression pattern of TRAP α RNA was studied under several experimental conditions. The induction profile of this gene by GM-CSF stimulation was examined. As expected, after deprivation and re-addition of GM-CSF, mRNA levels of TRAP α in the TF-1 cells increased (Fig. 3a), verifying that this gene is inducible. Notably, the induction was more obvious for the shortest RNA species, which increased by 24 h. To see if the expression level changes during the cell cycle, the MG-63 osteosarcoma cell line was examined because it stops growing when either cultured under reduced serum conditions or confluent. In our cell cycle analyses using this cell line, reduction of the serum concentration to 0.5% resulted in increased G1 phase cells and decreased S and G2/M phase cells by day two compared to the control condition with 10% serum (Fig. 3c, 12 and 13). The G2/M phase cells further decreased by day seven, suggesting that the cells were arrested at the G1/S checkpoint (Fig. 3c, 14). Similarly, the MG-63 cells that were confluent for 7 days were mostly in the G1 phase (Fig. 3c, 15). When the corresponding RNA samples from the MG-63 cells were analyzed, expression of TRAP α mRNA gradually decreased in the low serum condition (Fig. 3b, lanes 8–10) and was even less in the confluent cells (Fig. 3b, lane 11). These findings suggest that this gene is downregulated in quiescent cells. Also in the MG-63 cells, the change in the RNA level was most obvious for the shortest band.

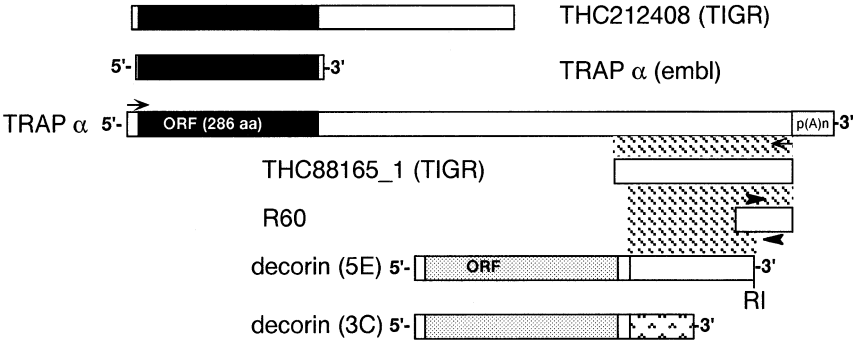


Fig. 2. Diagram showing the relationship of previously reported decorin cDNA to the TRAP α cDNA and related ESTs. The shaded area between sequences indicates identity between the sequences. TRAP α and decorin ORFs are represented by filled and dotted boxes, respectively. The primers used in the initial RACE reactions and subsequent amplification of the full length cDNA are depicted by arrowheads and arrows, respectively. Two published 3′-ends of decorin cDNA are distinguished by a pattern filling the region. The accession number of the previously published TRAP α sequence (shown as TRAP α (embl)) is Z12830. The decorin sequences are from Krusius et al. [22]. An initial search of the TIGR database with R60 found THC88165_1, searching the database with Z12830 returned THC212408.

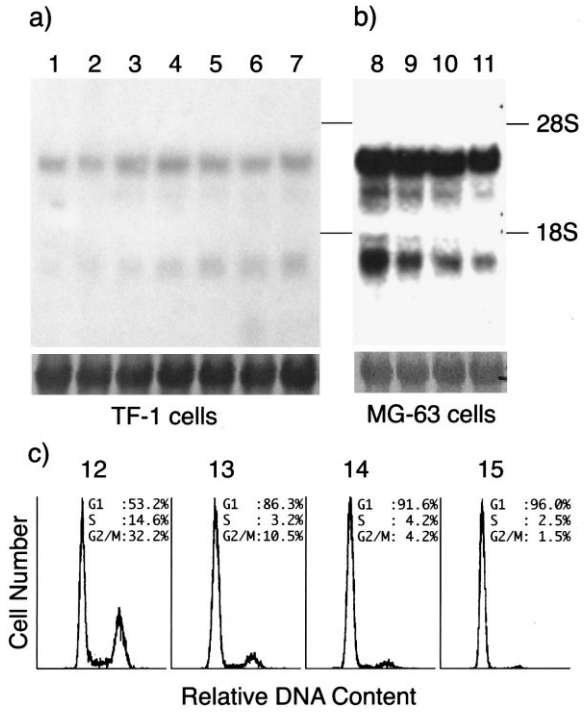


Fig. 3. Expression of the TRAP α gene. (a and b) A 1.5 kb cDNA fragment encompassing the whole ORF of TRAP α was labelled and hybridized to Northern blots (upper panels). Lower panels show 18S ribosomal RNA of corresponding blots stained with methylene blue. (a) Induction profile of the TRAP α mRNA after GM-CSF addition. RNA from cells before (lane 1) as well as 2, 4, 8, 24 and 48 h after GM-CSF addition (lanes 2–6, respectively) and from growing cells (lane 7) were analyzed. (b) RNA from the MG-63 cells in their rapidly growing state (lane 8), kept 2 and 7 days in medium with 0.5% serum (lanes 9 and 10, respectively) and kept confluent for 7 days (lane 11) were analyzed. (c) Cell cycle analyses of MG-63 cells. The cells were treated as in b and analyzed by flow cytometry. Panels 12–15 correspond to lanes 8–11 in b, respectively. Calculated proportions of cells in each phase of the cell cycle are shown.

3.4. Analyzing variant transcripts

As the R60 probe, which represented the 3′-end of the cDNA, hybridized only with the largest 3.3 kb band (data not shown), the smaller bands detected with the 1.5 kb probe might represent either related but distinct sequences, alternative polyadenylation or splicing of the mRNA that removes the R60 sequence. The TRAP α transcripts were, therefore, examined further. We performed a 3′-RACE reaction using a primer designed to anneal to the 5′-untranslated region of the TRAP α cDNA. The reaction gave five distinct bands (Fig. 4a), the largest of which matched the size of our full length cDNA. Taking into account the length of a poly(A) tract, the approximate sizes of these fragments matched those of the observed bands in the Northern blot analyses. The sequences of the cloned 3′-RACE fragments suggested a complex pattern of alternative polyadenylation of this gene. The full length transcript had an atypical polyadenylation signal sequence (AAUAAU) which was 30 bases upstream from the beginning of the poly(A) tail. The clones derived from the shortest band did not have the poly(A) tail and were not analyzed further. Each of the remaining bands gave clones with slightly different polyadenylation sites and hence sizes, with variations of the polyadenylation signals (Fig. 4b, double underlines). When compared to the full length cDNA sequence, these clones from the three bands all were predicted to have a U-rich sequence downstream of the poly(A) site, which is another requisite of a polyadenylation event [24] (Fig. 4b, dotted underlines).

3.5. RH mapping

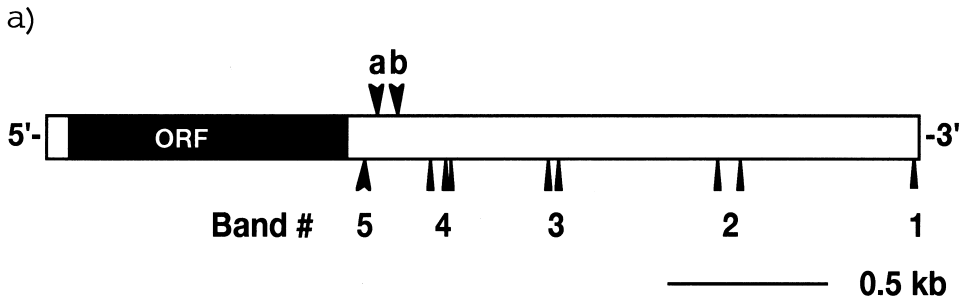
We performed PCR-based radiation hybrid mapping to locate chromosomally the human TRAP α gene. We found the nearest neighbor of the TRAP α gene to be WI-4041, which is assigned to the short arm of the sixth chromosome at 60 cR from the telomere.

4. Discussion

Previously reported canine and human TRAP α cDNA sequences did not have a poly(A) tail nor an authentic polyadenylation signal [25,26], quite similar to the shortest of our 3′-RACE product. In addition, our initial attempt to screen a human cDNA library with the R60 probe also yielded clones almost identical to R60 itself. These facts indicate a factor intrinsic to the TRAP α gene hampering the cloning of the full length cDNA by conventional methods. Utilizing the RACE method, we successfully amplified the full length cDNA for TRAP α. The sequence of the cDNA shows a long 3′-untranslated region quite rich in short stretches of homopyrimidines, which might predispose to the formation of stem loops and other secondary structures. Our PCR reactions used highly stringent conditions and invariably yielded a clear single band, which underscores the authenticity of our cDNA.

Nearly every metazoan mRNA has a polyadenylation signal sequence 10–30 bases upstream of the actual polyadenylation site [24]. The signal sequence, AAUAAA, is strictly conserved to be efficient, with the only exception being AUUAAA, resulting in 77% activity compared to the canonical sequence [27]. Other types of substitutions are rare and significantly reduce the polyadenylation efficiency [27]. Strikingly, none of the predicted polyadenylation signals in the TRAP α transcripts were canonical. The shortest 1.2 kb band contained the AUUAAA hexanucleotide, which might explain the relative abundance of the shortest band in our Northern blot analyses. Alternative polyadenylation in other genes sometimes results in transcripts with an altered half-life or intracellular localization [28]. Possibly, TRAP α might exploit the differential efficiency of these polyadenylation signals to generate transcripts of different 3′-structures and hence fates. Indeed, the induction of the TRAP α gene was most obvious for the shortest band in our Northern blot analyses (Fig. 3). Compared with the previous examples of alternative polyadenylation [28], TRAP α is a unique entity in that it utilizes arrays of signal sequences that are exclusively non-canonical

TRAP α was initially isolated as a protein that exists within a crosslinkable distance from the amino-terminal signal sequence of selected secreted polypeptides during their co-translational translocation into the microsome [25,29]. Although antibodies directed against TRAP α were inhibitory to the translocation process [30], Migliaccio et al. subsequently showed that the protein was unessential for this process [31]. TRAP α is a type I membrane protein residing in the membrane of the ER and the nuclear envelope [25,32]. It binds calcium in vitro [33]. Otherwise, the function of this



b)

Band#	Length	Sequence
1	3,267	TGAGAATAATGTGTTCATAAAGCATGGATCTCGTAAAAAAA---
2	2,243	AGACAAAAAAACAAAACGAAATACACACAGAAAAAAGTCTTTCCAAACTGTTTAAG---
	2,161	TGGCATTATTGTCAGACTGGATGGAGGTGAAGTAAAATAGTATGAACAATTAAGAGGCTCTCCCCCTCTTGTCTTTAA--
3	1,686	CAAAAAGAAACTAGTACATTTGTGAAAATTGTGTTGTGTTGTCCATTGTTGC---
	1,663	CTTTAATATATGAAACCTAATCCTACCCCCTTTTTTAACAAAAAGAAACTAGTACATTTGTGAA---
4	1,296	ACTAAATATAATGCTGTTGTCTCTTCCTTTTTGACATTTTCTGATTTTTTCCCCCAAAACT---
	1,273	CTGAAACAAGTCTTTAGAATACTAAATATAATGCTGTTGTCTCTTCCTTTTTGACATTTTCTG---
	1,206	AACCATTAAAATCTATGTGTGTGCGTGTGTATGTATGTGAGCAGTTGGTCTTACCAGAATCATTGTTGAA---

Fig. 4. Alternative polyadenylation of the TRAP α gene. (a) Diagram showing the ends of cDNAs. cDNA ends followed by poly(A) tails were clustered in four regions (including the full length cDNA) and are shown by small arrowheads. cDNA ends without poly(A) tails are shown by fat arrowheads. The reported cDNA ends of Z12830 and X51367, the canine homologue, lack the poly(A) tails and are indicated by arrowheads with a and b, respectively. In (b), predicted polyadenylation signals, the last bases before the poly(A) tail and the U-rich downstream sequences are denoted by double, single and dotted underlines, respectively. Sequences are of the full length cDNA to show the U-rich sequences downstream of the polyadenylation sites. In some cases, the exact point of cleavage is obscured due to intrinsic poly(A) tracts on the full length sequence, the first As of such poly(A) tracts are tentatively assigned as the last base before the poly(A) tail.

protein has remained unexplored. To our knowledge, induction of an ER-resident membrane protein by GM-CSF is a novel finding. In factor-stimulated cells, the protein synthesis might reach a rate exceeding that of protein maturation in the ER, resulting in the accumulation of improperly folded proteins and subsequent an unfolded protein response (UPR) [34]. The induction of TRAP α might thus relate to the UPR. On the other hand, the induction might well be dependent on pathways that are already known to be expedited by GM-CSF. Recently, Brewer et al. have hypothesized that growth factors regulate the steady-state level of molecular chaperones through a pathway distinct from UPR [35], wherein the induction kinetics were of the delayed early response type, similar to that of TRAP α by GM-CSF. Investigating the transcriptional elements that govern the expression of TRAP α definitely would tell us more about the detailed mechanism of its induction.

Finally, our radiation hybrid mapping experiment localized the TRAP α gene on the short arm of human chromosome 6, the nearest marker being WI-4041. Interestingly, the genetic susceptibility locus for schizophrenia has been mapped very close to this marker [36–38]. A marker D6S296, to which the highest lod score has been assigned in one study [37], is mapped only 3 cR centromeric from WI-4041 according to WICGR Mapping Service. Future studies will examine if TRAP α is altered in schizophrenic patients.

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