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Repression of A $TAF_{II}32$ Isoform as Part of a Program of Genes Regulated during Mpl Ligand-Induced Megakaryocyte Differentiation

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Circulating platelets, essential for thrombosis and hemostasis, originate from megakaryocytes. Megakaryocyte growth, differentiation and survival processes are regulated by the c-Mpl receptor ligand. In the current study we used differential display to identify part of the program of genes regulated during Mpl ligand-induced murine megakaryocyte differentiation. Several of the genes, including the retinoblastoma binding protein p84, were found to be induced, while others were repressed. One such repressed gene was identified as a TATA-binding protein (TBP)-Associated Factor (TAF) family member, $TAF_{II}32$, previously reported to be upregulated during apoptosis. Our analysis of various cell types suggested that the previously identified species homologs, human $TAF_{II}32$ and murine $TAF_{II}32$, are in fact different isoforms, which we propose to re-name $TAF_{II}32\alpha$ and $TAF_{II}32\beta$, respectively. Only the $TAF_{II}32\beta$ isoform is regulated during Mpl ligand-induced megakaryocyte differentiation, which suggests individual roles for the two forms.

In 1990, the proto-oncogene *c-mpl*, was discovered as the cellular homolog of the viral oncogene *v-mpl* from the myeloproliferative leukemia virus (1). Since then, c-Mpl , the protein encoded by *c-mpl*, has been identified as a member of the hematopoietic growth factor superfamily, a heterogeneous group of receptors, with roles in survival, proliferation and differentiation of hematopoietic cells. The search for a humoral factor capable of stimulating c-Mpl, resulted in the discovery of Mpl-ligand (also referred to as thrombopoietin (TPO), and Megakaryocyte Growth and Development Factor (MGDF)) as the native ligand for c-Mpl (2–4). Targeted studies by others have identified a link between c-Mpl stimulation and the regulation of megakaryocyte gene expression, such as, NF-E2 (5). In our current study, we have taken advantage of a subclone (Y10) of the L8057 murine megakaryoblastic cell line (6), which is capable of expressing differentiation markers in response to MGDF treatment (7). To analyze some of the molecular effects of c-Mpl stimulation during this process, we employed a differential display RT-PCR approach (8) to Y10 cells treated or untreated with MGDF at three separate time points (2, 8 and 24 hours). In total, thirty individual differentially expressed clones were obtained from twenty-four primer sets. In the current study, we have concentrated on displayed cDNA fragments, whose expression was confirmed by Northern blot analysis to be upregulated or repressed following treatment with MGDF. In particular, we focused on clone R6 which was displayed as reduced, the encoded protein of which, $TAF_{II}32$, has been intimated to be a contact point for signaling events that affect the basal transcription machinery (9, 10), as well as having a role in neuronal cell apoptosis (11). Our data indicate that an early response to MGDF treatment involved the simultaneous modulation of expression of a cluster of genes, whose significance for megakaryopoiesis awaits investigation. A challenging future goal would be to explore whether there exists a Mpl ligand-responsive master switch gene or if diverse Mpl ligand-mediated signaling pathways control the expression of different genes.

MATERIALS AND METHODS

Cell culture, RNA isolation and Northern blot analysis. Y10 cells were maintained in F12 Medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-Glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (all GIBCO BRL Life Technologies, Grand Island, NY), in a humidified incubator at 37°C with 5% CO_2. Sixteen hours prior to the experiment, they were re-seeded at a concentration of 2×10^5 per ml in Iscove's modified Dulbecco's medium (IMDM) supplemented as above. Viable cells were counted using a hemocy-

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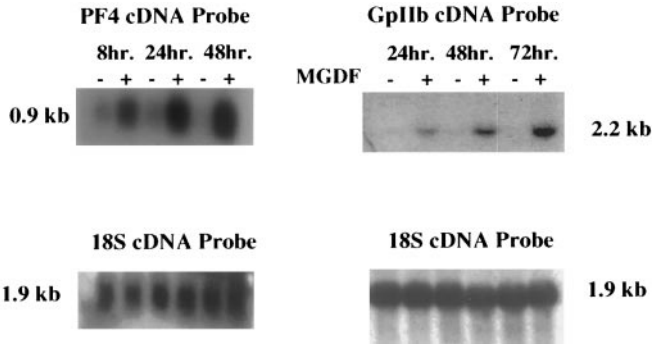

FIG. 1. Differentiated Y10 cells show increased levels of PF4 and GpIIb mRNAs within eight hours of treatment with MGDF. Northern blot analysis was carried out on total RNA samples (20 μg) extracted from vehicle- or MGDF-treated Y10 megakaryocytes for the indicated times. Radiolabeled cDNAs of Platelet Factor 4 (PF4) or Glycoprotein IIb (GpIIb) were used in the hybridization reaction. Equal loading of RNA was confirmed by hybridization with 18S ribosommal cDNA. Blots were washed under high stringency conditions, as detailed under Materials and Methods.

tometer in conjunction with Trypan Blue exclusion (GIBCO BRL). A recombinant form of Mpl ligand, pegylated-recombinant human megakaryocyte growth and development factor (abbreviated as MGDF) (generous gift of Amgen Inc. Thousand Oaks, CA) was used where indicated. MGDF was added to the Y10 cell culture for 2, 8, 24, 48 or 72 hours as indicated, at a concentration (25 ng/ml) previously shown to be maximal for differentiation of this cell line (7). Total RNA was extracted using Trizol reagent (GIBCO BRL) according to manufacturer's specifications. Northern blot analysis was performed as described previously (12). cDNA probes used, in addition to the differentially displayed fragments, were either obtained by RT-PCR of appropriate tissue, or obtained from other investigators. High stringency washing is defined as 0.2× SSC (13), 0.1% sodium dodecyl sulfate (SDS) (American Bioanalytical, Natick, MA) at 65°C for 20 minutes, while low stringency washing is defined as 1× SSC, 0.1% SDS at 55°C for 20 minutes. HeLa cells (American Type Culture Collection (ATCC), Mannassas, MD), were maintained in media as instructed by the ATCC. MCF-7, MCF-10F and Hs-578T cells were maintained in media as previously described (14, 15).

Differential display reverse transcriptase-polymerase chain reaction. DNase-treated total RNA (MessageClean Kit, Genhunter, Nashville TN) from MGDF-treated or untreated Y10 cells was used as the source for differential display RT-PCR, which was performed by the method of Liang *et al.* (8), in accordance with the RNAimage kit (Genhunter, Nashville, TN). cDNAs were amplified using the same anchored oligo-dT primer used for reverse transcription (H-T11A, C, or G) in association with one of eight arbitrary sense primers (H-AP) supplied by the RNAimage Kit. Following the PCR reactions, the radiolabeled products (3.5 μl) were separated on a 6% denaturing polyacrylamide sequencing gel and electrophoresed at a constant power (75 Watts). Differentially expressed cDNAs, identified by autoradiography, were extracted from the gel. The DNA was then eluted from the gel fragment and a reamplification PCR reaction was then carried out. Next, the identification and size of reamplified products were verified by electrophoresis on a 1.5% ethidium bromide agarose gel, before the cDNA fragments were subcloned into TA vector (Invitrogen, Carslbad, CA) for either DNA sequencing or Northern blot analysis.

DNA sequencing and homology searches of differentially expressed clones. Plasmid DNAs obtained from the transformed *E. coli* above were sequenced by the dideoxy chain termination method (16), using the dsDNA Cycle Sequencing System (GIBCO BRL). The primer used was either the M13/pUC Forward 23-Base primer or the M13/pUC Reverse 17-Base primer, or both sets of primers when the insert was greater than 250 base pairs. Sequences of clone inserts were then analyzed for homology to known genes in GenBank, EMBL, DDBJ and PDB databases, using the Basic local alignment search tool (BLAST) algorithm (17).

Reverse-transcription of mTAFII32 mRNA. The coding region of the mouse $TAF_{II}32$ cDNA was derived from Y10 megakaryocytes. To this end, proliferating cells (2×10^5/ml) were collected and total RNA was extracted (see above) following Trizol-treatment, according to the manufacturer's specifications. First strand cDNA was obtained using the ThermoScript RT-PCR system (GIBCO BRL) using oligo-dT primers. The PCR amplification reaction was carried out using DNA primers that were designed on the rat sequence

TABLE 1

Sequence Analysis of Several cDNA Species Regulated by MGDF Treatment of Y10 Megakaryocytes

Subclone[a]	% Sequence identity (over # of bases)	Description of homology	Northern blot analysis[b] (fold change/mRNA size)	Genbank accession no.	Reference
I2	N.H.[d]	No Homology	INDUCED (2.0/1.8 kb)	A1738402	—
I9	87 (155)	Human Rb Binding p84 nuclear matrix protein (nt. 209–363)[c]	INDUCED (2.2/2.3 kb)	L36529	(24)
I17	90 (490)	Gp55 glycoprotein of Friend Leukemia Virus (nt. 5570–6218[c]	INDUCED (1.8/4.6 kb)	K00021	(25)
I20	N.H.[d]	No Homology	INDUCED (1.5/1.2 kb)	A1738403	—
I22	N.H.[d]	No Homology	REPRESSED (1.5/3.8 kb)	A1738404	—
R6	91 (235)	Rat TBP-Associated Factor $rTAF_{II}32$ (nt. 1176–1405)[c]	REPRESSED (2/2.4 kb)	U40188	(11)
R12	N.H.[d]	No Homology	INDUCED (1.8/4.8 kb)	A1738401	—

[a] Subclones denoted by numbers, based on the original screening (I = Induced and R = Repressed).
[b] Northern blotting of RNA samples collected from Y10 cells treated with MGDF for 2, 4, 8 and 24 hours repeated 2–3 times (as in Fig. 2). Fold induction or repression is indicated in parentheses.
[c] nt. = nucleotide position in relation to the translation initiation site.
[d] DNA sequences of clones for which no homology was found (N.H.) were deposited in GenBank.

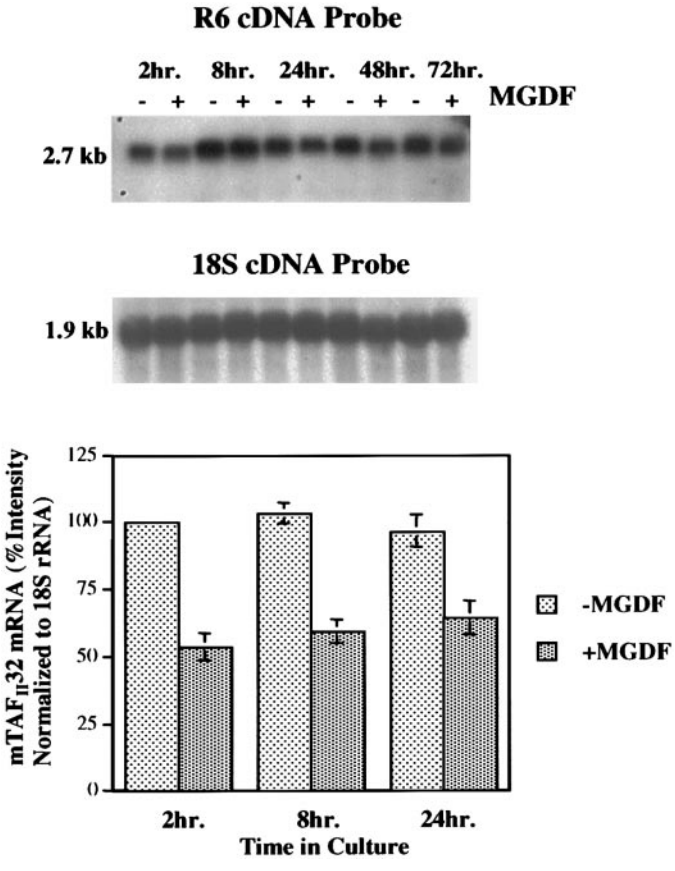

FIG. 2. Qualitative and quantitative analysis of mRNA expression of Clone R6 in Y10 megakaryocytes treated or untreated with MGDF. Aliquots of total RNA (20 μg) prepared from Y10 megakaryocytes, treated or untreated with MGDF for the indicated times, were analyzed by Northern blot analysis. cDNA from a differentially expressed clone (R6) was radiolabeled and hybridized to the Northern blot membranes, showing a 2.7 kb band. These blots were washed under high stringency conditions, as detailed under Materials and Methods. In the lower panel, values obtained from an Electrophoresis Documentation and Analysis System (Eastman Kodak, Rochester, NY) of three independent experiments and Northern blots, were plotted. Data are averages ± standard deviations. Equal loading of RNA was confirmed by hybridization with 18S ribosommal cDNA.

previously published in GenBank (U40188) i.e. sense 5′-GCTGCAAGATGGCGCCTCTCAAGAA-3′, and anti-sense 5′-GAGACCAGGAACTGAAATCCAAGTG-3′. The product obtained was subcloned to TA vector (Invitrogen) and subjected to DNA sequencing (16) to confirm over 95% identity with rat TAF$_{II}$32 cDNA (11).

RESULTS AND DISCUSSION

The Mpl ligand has been shown to be capable of eliciting proliferative, differentiative and survival effects on a variety of hematopoietic cell types, including the most primitive CD34+ cells (18–20). To investigate the program of genes regulated by the this cytokine during megakaryocyte differentiation, a cell culture system was employed. The Y10 cell line, subcloned from the L8057 murine megakaryoblastic cells (7), was selected due to its ability to differentiate in the presence of the recombinant form of Mpl ligand MGDF, as measured by increased acetylcholinesterase activity within 24 hours of treatment (7). Further analysis of this cell line in the current study revealed increased expression of the lineage-specific markers platelet factor 4 (PF4) and GpIIb mRNA as early as 8 hours following MGDF treatment (Fig. 1). These results defined a period during which MGDF mediated gene expression was altered.

To analyze in an unbiased manner some of the MGDF-regulated molecular events involved during Y10 cell differentiation, we employed a differential display RT-PCR approach (8) to MGDF treated and untreated Y10 cells using three separate time points (2, 8 and 24 hours). In total, thirty individual differentially expressed clones were obtained from twenty-four primer sets (data not shown). Of these thirty clones, the majority (73%) showed no homology to known genes submitted to GenBank. When the cDNA fragments were utilized as probes for Northern blot analysis, 43% of the clones hybridized with sufficiently expressed species, of which 38% verified the original differentially expressed profile (Table 1). This indicated that a program of gene expression was initiated by MGDF treatment of Y10 megakaryocytes during the period analyzed. One of these cDNA species, clone R6 (235 bp), was selected as encoding a message repressed

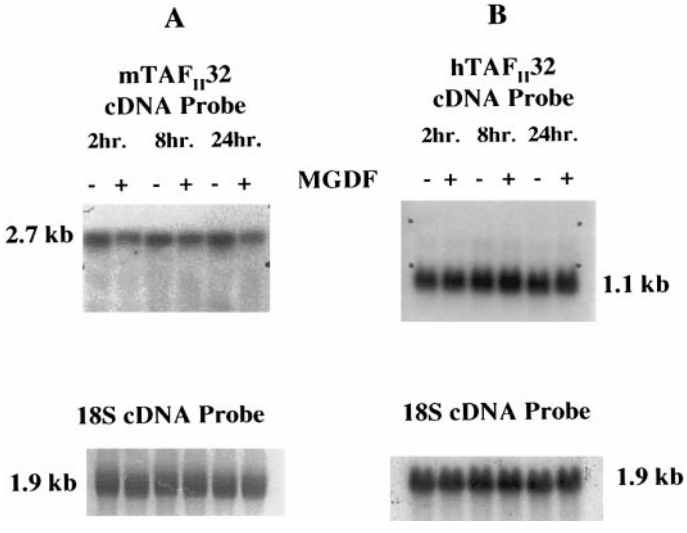

FIG. 3. Y10 megakaryocytes express two mRNA isoforms of TAF$_{II}$32, of which only the 2.7 kb moiety is repressed by MGDF treatment. Northern blots of RNA samples (20 μg) from MGDF-treated or untreated Y10 megakaryocytes over time, were hybridized with either human TAF$_{II}$32 (hTAF$_{II}$32) (A), or murine TAF$_{II}$32 (mTAF$_{II}$32) (B) full-length cDNAs. Membranes were washed under high stringency wash conditions, as detailed under Materials and Methods. Equal loading of RNA was confirmed by hybridization with 18S ribosommal cDNA. The data are representative of three experiments.

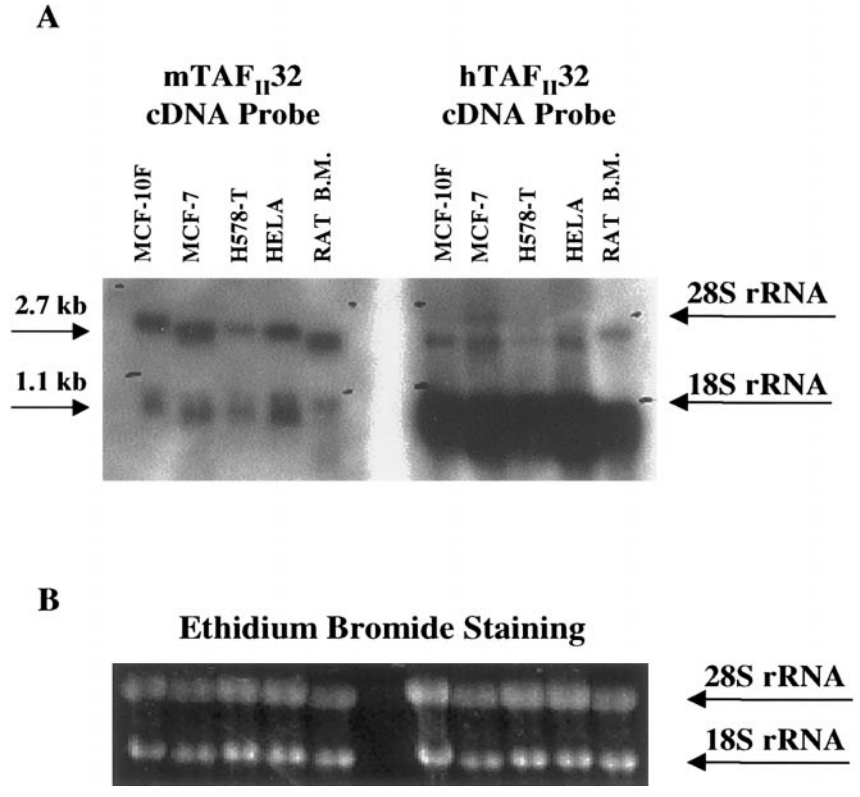

FIG. 4. Human transformed and non-transformed cell lines, as well as rodent primary cells and cell lines express two isoforms of TAF$_{II}$32, as shown by Northern blot analyses. (A) Northern blots of RNA samples (20 μg) from multiple human cell lines (MCF-10F, MCF-7, H578-T and HELA), and rat primary bone marrow cells (B.M.) were hybridized with either human TAF$_{II}$32 (hTAF$_{II}$32) or murine TAF$_{II}$32 (mTAF$_{II}$32) full-length cDNAs. The arrows indicate the positions of the 18S and 28S rRNAs. (B) Equal loading of RNA was confirmed by ethidium bromide staining of the ribosomal bands. The blots were washed under low stringency conditions, as detailed under Materials and Methods. The data are representative of three experiments.

as early as 2 hours following MGDF treatment. This was confirmed by detecting, with R6 cDNA, a 2.7 kb mRNA repressed in a Northern blot assay (Fig. 2). This clone, R6, was further analyzed and identified as murine (m)TAF$_{II}$32, based on over 90% nucleotide identity with rat (r)TAF$_{II}$32 (GenBank Accession #U40188). The rTAF$_{II}$32 mRNA species had previously been shown to be upregulated in the PC12 neuronal cell line, coincident with the onset of growth factor withdrawal-induced apoptosis (11). Like the other members of the cytokine family, Mpl ligand has been implicated as a cell survival factor (21). It was of note therefore, that in differentiating Y10 megakaryocytes the R6 clone was repressed by MGDF.

A full-length murine TAF$_{II}$32 cDNA (1.1 kb) was obtained via RT-PCR, using primers directed to the published sequence, as described under methods. This cDNA was subsequently used to verify the repression profile exhibited by clone R6 on Northern blots of MGDF-treated Y10 cells washed under high stringent conditions (Fig. 3A). Concurrently, the human homologue of rTAF$_{II}$32 cDNA was obtained and assayed for cross reactivity in Y10 megakaryocytes using the same degree of stringency of membrane washing (Fig. 3B). Interestingly, only the 2.7 kb mRNA species (detected by mTAF$_{II}$32 cDNA), but not the 1.1 kb species (detected by hTAF$_{II}$32 cDNA), showed the repressed expression pattern observed with clone R6 following treatment of the cells with MGDF. Total RNA samples obtained from a variety of transformed, non-transformed and primary tissues from human and rodents showed expression of both TAF$_{II}$32 species in the same cell type, by Northern blot analysis subjected to low stringency conditions (Fig. 4). Surprisingly, the hTAF$_{II}$32 cDNA probe, not only hybridized to a 1.1 kb RNA species that was consistent with the published hTAF$_{II}$32 mRNA (22), but also cross reacted with the murine species (2.7 kb). For this reason and for ease of classification, we suggest that the originally discovered 1.1 kb human TAF$_{II}$32, be renamed TAF$_{II}$32α, and the MGDF-regulated 2.7 kb form be renamed TAF$_{II}$32β. Our results indicate that, in contrast to the previously published reports (11, 22), the rat and human TAF$_{II}$32s are not species homologs but rather two different isoforms often co-expressed in the same cell. This observation is supported by the recently reported observation of two separate genes for TAF$_{II}$32 (23) in human tissue.

Previous reports have indicated that each of the $TAF_{II}32$ mRNAs were ubiquitously detected in all the major tissue types, with slightly more abundance in heart tissue (11, 22). Our studies indicate that $TAF_{II}32\alpha$ is expressed at extremely high levels in rodent primary BM cells, fibroblastic and megakaryocytic cell lines as well as human epithelial cell lines. In contrast, $TAF_{II}32\beta$ (the 2.7 kb mRNA species) was highly abundant in megakaryocytic and primary BM cells and less in other cell types. Indeed, Aoki *et al*. (11) were able to detect the 2.7 kb $TAF_{II}32$ mRNA species in adult tissues, only following poly-A^+ selection of total RNA. A possible explanation for the high expression of both $TAF_{II}32$ isoforms in BM samples is that, unlike most other tissue in the body, BM cells are a heterogeneous population of terminally differentiated and proliferating cells which have a relatively short lifespan (days). If, as we suggest, $TAF_{II}32$ is primarily involved in maintaining a proliferation profile, then it would explain the higher expression level in primary BM cells, than in a terminally differentiated tissue such as liver or heart. The different relative levels of expression of $TAF_{II}32\alpha$ and $TAF_{II}32\beta$ in different cells further suggest that these isoforms may play distinct roles in these cells. In the current study, the sustained MGDF-induced repression of $TAF_{II}32\beta$ may be required for a slowing down or pausing of the cell cycle to facilitate Mpl ligand-induced differentiation. Future experiments will aim to explore the potential role(s) of the Mpl ligand-regulated $TAF_{II}32\beta$ in megakaryocyte development, and of the other displayed genes identified in this study (24, 25).

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