Alternative Splicing of the Human Cyclin D-Binding Myb-Like Protein (hDMP1) Yields a Truncated Protein Isoform that Alters Macrophage Differentiation Patterns

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Running title: hDMP1 alters macrophage differentiation
SUMMARY

We have cloned two novel, alternatively spliced messages of human cyclin D-binding myb-like protein (hDMP1). The known, full-length protein has been named hDMP1\(a\) and the new isoforms hDMP1\(b\) and hDMP1\(g\). The hDMP1\(a\), \(b\), and \(g\) splice variants have unique expression patterns in normal hematopoietic cells; hDMP1\(a\) mRNA transcripts are strongly expressed in quiescent CD34\(^+\) cells and freshly-isolated peripheral blood leukocytes, as compared to hDMP1\(b\). In contrast, activated T cells and developing myeloid cells, macrophages and granulocytes express low levels of hDMP1\(b\) transcripts and hDMP1\(g\) is ubiquitously and weakly expressed. Mouse Dmp1 has been shown to activate CD13/aminopeptidase N (APN) and p19\(_{\text{ARF}}\) gene expression via binding to canonical DNA recognition sites in the respective promoters. Assessment of CD13/APN promoter responsiveness demonstrated that hDMP1\(a\) but not hDMP1\(b\) and \(g\) is a transcriptional activator. Furthermore, hDMP1\(a\) was found to inhibit the CD13/APN promoter transactivation ability of hDMP1\(b\). Stable, ectopic expression of hDMP1\(b\), and to a lesser extent hDMP1\(g\), reduced endogenous cell surface levels of CD13/APN in U937 cells. Moreover, stable, ectopic expression of hDMP1\(b\) altered PMA-induced terminal differentiation of U937 cells to macrophages and resulted in maintenance of proliferation. These results demonstrate that hDMP1\(b\) antagonizes hDMP1\(a\) activity and suggest that cellular functions of hDMP1 may be regulated by cellular hDMP1 isoform levels.
INTRODUCTION

The 761-amino-acid mouse cyclin D-binding myb-like protein (mDmp1) transcription factor was first identified using cyclin D2 as bait in a yeast two-hybrid screen and human DMP1 was cloned shortly thereafter (1,2). Both mouse and human DMP1 are composed of a central domain containing three myb-like repeats flanked by an acidic transactivation and a cyclin binding domain on the N terminus and an acidic transactivation domain at the C terminus (3). In humans, hDMP1 mRNA is ubiquitously expressed at low levels in normal human tissues, with highest expression levels in testis, spleen, thymus and peripheral blood leukocytes (2). Interestingly the hDMP1 locus is localized on chromosome 7q21, a region frequently deleted as part of the 7q-minus and monosomy 7 abnormalities of acute myeloid leukemia and myelodysplastic syndrome. DMP1 binds to nonameric Ets consensus DNA sequences containing G-G/T-A cores, competes with Ets-family proteins, and has been shown to be a transcriptional activator (1). DMP1 binding sites are found in the promoters of two well-known genes, CD13/aminopeptidase N (APN) and the mouse tumor suppressor p19ARF (also referred to as ARF or p14ARF in humans) which is encoded from the Ink4a-Arf locus (4).

DMP1 has been shown to be present in non-dividing cells and has a role in cell differentiation in certain hematopoietic lineages (1,3), and is critical for cell cycle control via regulation of ARF (5). One such gene regulated by DMP1, the ectoenzyme CD13/APN, has been implicated in myeloid development (6). CD13/APN is a membrane bound metalloproteinase that is expressed on normal human myeloid and lymphoid cells (7,8), as well as non-hematopoietic cells (9). CD13/APN expression is developmentally regulated (10), functions in peptide degradation, cell-cell adhesion (11,12), and participates in growth and development of both
hematopoietic and endothelial cells reviewed in Riemann et al. (10). A direct role for CD13/APN has been proposed for human dendritic cell development in culture (13). In myeloid cells it has been shown that c-Myb, Ets family members and mDmp1 regulate CD13/APN transcription (6). Activation of CD13/APN gene expression is enhanced by a cooperative interaction between c-Myb bound to its cognate site and mDmp1 bound to one of the three downstream GGA core sites (6). Thus, CD13/APN promoter activity requires the intact DNA binding and transactivation domains of mDmp1, since mutants disrupted in either domain are biologically inert. Intriguingly, that c-Myb and mDmp1 show synergistic effects in activating the CD13/APN promoter implies that two different Myb family proteins collaborate in regulating CD13/APN gene expression and points to an important role for DMP1 in normal myeloid cell development (6).

Herein, we describe two new splice variants of human DMP1 message generated by alternative splicing through the use of two different splice acceptor sites. We propose that the previously cloned hDMP1 message (2) be termed hDMP1[α] and the new splice variants hDMP1[β] and [γ]. Distinct patterns of hDMP1[α], [β], and [γ]-mRNA transcript expression in primary myeloid and lymphoid cells during in vitro development led us to consider the possibility that alternative hDMP1[β] and [γ]-isoforms might have distinct cellular functions as compared to hDMP1[α]. We confirm a role for the original DMP1 isoform, hDMP1[α], in activation of CD13/APN gene expression (6) and document antagonistic functional properties for the hDMP1[β]-isoform in CD13/APN regulation using CD13/APN promoter-reporters and through stable, ectopic hDMP1[β]- and [γ]-isoform expression in the U937 myeloid cell line. Moreover, stable, ectopic expression of hDMP1[γ] in U937 cells altered PMA-induced terminal differentiation to macrophages.
EXPERIMENTAL PROCEDURES

Cell isolations- Peripheral blood mononuclear cell (PBMNC) samples were obtained from healthy donors recruited by the General Clinical Research Center at Green Hospital, La Jolla, CA, and cord blood samples from placentas acquired from mothers with normal, full term deliveries at Scripps Memorial Hospital, La Jolla, CA. Protocols and the use of all human samples were approved by the IRB of The Scripps Research Institute. CD34+ cells from cord and peripheral blood as well as PBMNC and neutrophils from peripheral blood were isolated as previously reported (14,15).

RT-PCR reactions- Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA from 0.5-1 µg of sample RNA was generated (oligo dT primers, Roche Molecular Biochemicals, Indianapolis, IN; M-MLV Reverse Transcriptase, Promega, Madison, WI). CD34+ RNA was prepared from 2x10⁴ cells and cDNA was generated using Superscript™II reverse transcriptase (Invitrogen, Carlsbad, CA). The relative expression of the hDMP1 splice variants b and g as well as the previously cloned hDMP1a was determined by RT-PCR using primers encompassing the found insertions (Fig. 2A, forward primer: 5’-TACAGGACTATAGCATGGGGTC-3’; reverse primer: 5’-ACTTCCCTGTGTTGCAAGTATC-3’). Conditions for PCR amplification were: One cycle for 5 min at 94°C, 35 cycles with 15 s at 94°C, 30 s at 58°C, 60 s at 72°C and a last cycle at 72°C for 7 min. HDMP1b and g were specifically amplified using the same forward primer as above but a b and g specific reverse primer (5’-CCATTTGACTGGTTTGGAAGTTG-3’) in the unique DNA insertion of these splice variants. PCR conditions were the same as above but using 32 cycles. GAPDH PCR was performed as described (16).
Cell culture – Cord blood CD34+ cells were differentiated in culture to either neutrophils or monocytes during a 15-day period with combinations of human recombinant cytokines. Initially, CD34+ cells were cultured in 10% BIT 9500 (StemCell Technology, Vancouver, Canada) supplemented serum-free IMDM medium containing hrSCF (300 ng/ml), hrG-CSF (10 ng/ml), hrIL-6 (10 U/ml), hrGM-CSF (50 ng/ml) and hrFL (300 ng/ml) to expand primitive progenitors. Growth factors were purchased from PeproTech (Rocky Hill, NJ USA). All cultures were maintained in a 37°C humidified incubator, containing 5% CO2. After four days of culture the cells were removed, washed, and the medium was changed to Myelocult™ H5100 (StemCell Technology, Vancouver, Canada) containing hrSCF (300 ng/ml), hrGM-CSF (10 ng/ml), hrIL-6 (10 U/ml) and hrIL-3 (10 ng/ml) to promote expansion of primitive myeloid progenitors. On day 10 of the cultures cells were removed, washed, and resuspended in either Myelocult™ hrG-CSF (10 ng/ml) for neutrophil development or Myelocult and hrM-CSF (5000 U/ml) for monocyte/macrophage development. Stage of development was assessed on cellular morphology and stage-specific cell surface markers using flow cytometry and appropriate antibody reagents (14,17,18). To obtain peripheral blood derived macrophages 2x10⁶ PBMNCs in 1 ml of RPMI containing 10% fetal bovine serum (FBS) (Hyclone, Ogden, UT), 2mM L-glutamine, and 100U/ml of penicillin and streptomycin (C-RPMI) were added to each well of a 24-well tissue culture plate and maintained overnight in a 37°C humidified incubator, containing 5% CO2. The next day non-adherent cells were removed by gentle washing and cells were then cultured in 1 ml of C-RPMI and 5000 U/ml of hrM-CSF.

The human U937 monocytic cell line was maintained in DMEM containing 10% fetal bovine serum (FBS) (Hyclone, Ogden, UT), 2mM L-glutamine, and 100 U/ml of penicillin and streptomycin (C-DMEM). U937 cells were differentiated using PMA (Sigma-Aldrich Corp., St.
Louis, MO). Briefly, PMA first was dissolved in DMSO at a concentration of $1.0 \times 10^{-4}$ M. For macrophage differentiation, PMA was diluted to concentrations as indicated in C-DMEM and cells were cultured at a density of $0.3 \times 10^6$ cells per ml for 1-4 days. All cultures were maintained in a 37°C humidified incubator containing 10% CO$_2$.

Non-adherent PBMNC were cultured in C-RPMI on 12-well plates pre-coated with anti-CD3 (1 µg/ml; Becton-Dickinson, BD Biosciences, San Jose, CA) and anti-CD28 (1 µg/ml; Becton-Dickinson) antibody for T-cell activation. Activation of T-cells was measured by FACS analysis of the CD69 surface marker (PerCP-conjugated CD69 antibody, Becton-Dickinson) after six days of incubation in a 37°C humidified incubator containing 5% CO$_2$.

*Expression plasmid and HIV vector construction and preparation of vector stocks- hDMP1* and its two variants were cloned by RT-PCR. Briefly, total RNA from KG-1 cells was reverse transcribed using M-MLV reverse transcriptase (Promega). The open reading frame of *hDMP1* was amplified with the Expand High Fidelity PCR System (Roche Molecular Biochemicals) and the following primers: Forward 5'-ATGAGCACAGTGGAAGAGGATTC-3' and reverse 5'-ATGACAGTTTACCAAATCTTC-3'. Blunt-ended fragments were 3' A-tailed with Taq DNA polymerase, purified, and ligated into the pcDNA3.1/V5-His-TOPO vector (Invitrogen).

Plasmid pCR-XL-CSPre was constructed by cloning the *MluI-Apal* fragment from the self-inactivating (SIN) HIV vector pHIV-SINPre (19) into the pCR-XL Topo backbone (Invitrogen). The CMV promoter from pcDNA3.1/V5-His-TOPO (*BglII-BamHI* fragment) was cloned into the *BamHI* site of pCR-XL-CSPre. The HIV vector VIPER was constructed by cloning a fragment containing the central polyurine tract (cPPT) (20), the human elongation factor five internal ribosomal entry site (IRES) (21), the truncated nerve growth factor receptor (tNGFR) and scaffold attachment region (SAR) sequence (22) between the *BamHI* and *SacII* of
pCR-XL-CSPre. HDMP1\(a\) and \(b\) were cloned into the unique \textit{BamHI} site of VIPER. The VSV-G-pseudotyped HIV vectors were generated by transient transfection using the selected transgene plasmid and the following third generation packaging plasmids: pMD.G (VSV-G), pMDLg/p.RRE (\textit{gag} and \textit{pol}), and pRSV-Rev (\textit{rev}) (23). The VIPER vector containing supernatant was harvested after 24 hours, concentrated by ultracentrifugation and resuspended in serum-free medium. HIV vector titers, defined as transducing units/ml (TU) were determined by transduction of 293T cells and flow cytometry analysis to determine the percentage of cells expressing tNGFR (14).

\textit{In vitro transcription and translation of hDMP1 isoforms}- Cell-free transcription and translation of hDMP1\(a\), \(b\), and \(g\) plasmid DNA was performed using the TnT T7-coupled reticulocyte lysate system (TnT\(^\circledR\) system) and the Transcend™ non-radioactive translation detection system according to the manufacturer’s instructions (Promega). A luciferase plasmid encoding a 61 kDa protein was used as positive control. The empty pcDNA3.1/V5-His-TOPO vector served as a negative control. To detect reaction products 15 \(\mu l\) were separated by SDS-PAGE, blotted and detected by chemiluminescence.

\textit{Dual luciferase reporter assays- Firefly luciferase} reporter plasmids containing a contiguous stretch of eight concatemerized DMP1-binding sites, pGL2-BS2, kindly provided by Charles J. Sherr, M.D, Ph.D., St. Jude Children’s Hospital, Memphis, TN or the myeloid CD13/APN promoter (pMyo), kindly provided by Jørgen Olson, M.D, University of Copenhagen, Denmark, were used for functional analysis of the different hDMP1 isoforms. For transfection experiments, 293T cells were grown to 70-80\% confluence in 60 mm\(^2\) dishes and transfected using a modified
calcium phosphate method (24); 6 µg of the reporter plasmids were co-transfected with 4 µg of the different hDMP1 expression vectors or the empty pcDNA3.1/V5-His-TOPO vector. Twenty ng of the pRL-TK Renilla luciferase vector were co-transfected in each experiment as an internal control for transfection efficiency. For mixing experiments, 4 µg of the reporter plasmids and 1.5 µg of pcDNA3.1-hDMP1 were co-transfected with varying amounts of pcDNA3.1-hDMP1 and The total amount of transfected DNA was adjusted to 11.5 µg with empty pcDNA3.1/V5-His-TOPO vector. Cell lysates from transfected cells were prepared 48h after transfection and luciferase activity was measured according to the manufacturer’s Dual-Luciferase™ reporter assay protocol (Promega). The ratio of Firefly to Renilla luciferase was calculated to obtain the relative luciferase activity (RLA). Each experiment was performed in triplicate and data are presented as mean ± S.E.M.

HIV VIPER transduction and cell sorting- For VIPER vector transduction 2x10⁵ U937 cells were added to round-bottom 96-well plates in 0.1 ml C-DMEM. Cells were transduced with VIPER control (tNGFR gene, no hDMP1 gene), VIPER (hDMP1) and (hDMP1) vectors at a multiplicity of infection (MOI) of 20 in the presence of 8 µg/ml polybrene. The plates were spun for 45 min at 1250 rpm at room temperature using a Sorvall RT 6000D centrifuge and then incubated for 4 hours in 10% CO₂ at 37°C. Cells were washed once and resuspended in fresh medium. The transduction was repeated the next day and cells were cultured for 10 days. After 10 days the upper 20% tNGFR expressing U937 cells were isolated on a FACS Vantage SE II cell sorter (Becton-Dickinson, Biosciences) using FITC-conjugated tNGFR antibodies.
Analysis of cell surface proteins- Flow cytometry was used to analyze expression of CD13/APN, CD11c and tNGFR on transduced U937 cells. In brief, 2x10^5 U937 cells in 100 µl of ice-cold FACS buffer were incubated with 1 µl of the FITC-conjugated CD13/APN (10M13, AMAC, Westbrook, ME), the FITC-conjugated CD11c (Leu-M5, Becton-Dickinson Biosciences) or the PE-conjugated NGFR antibody (C40-1457, Becton-Dickinson Biosciences), washed, and analyzed for flow cytometry or sorted. For antibody specificity, irrelevant FITC- and PE-conjugated mouse isotype controls (Beckman Coulter, Miami, FL) were used. For flow cytometry analysis 10^4 cell events per sample were acquired on a FACSCalibur machine (Becton-Dickinson Biosciences) running the Cell Quest acquisition software and analysis was accomplished using Cell Quest analysis software, version 3.4.

Proliferation assays- Proliferation was assessed by labeling cells with [3H]-thymidine. Differentiating U937 VIPER and VIPERb transfectants were resuspended in C-DMEM containing PMA at concentrations as indicated and seeded in 96-well plates. [3H]-thymidine was added for the last five hours of cell treatment (1 µCi/well). Cells were harvested at time points indicated using a Mach III cell harvester (Tomtec, Orange, CN) and [β]-radiation was detected on a Wallac 1450 MicroBeta Liquid Scintillation Counter (PerkinElmer, Boston, MA).

Morphological Evaluation – Morphological features of differentiated U937 VIPER and VIPERb cell were reviewed on cytospin slide preparations stained with May-Grunwald-Giemsa stain. Classically defined morphological features were used as indicators of macrophage differentiation including cell shape, adherence, ratio of cytoplasmic region to nuclei and cytoplasmic granulation.
RESULTS

Genomic organization and translation of the human DMP1 gene and its splice variants- During routine RT-PCR cloning for full-length hDMP1 message from the myeloid leukemic cell line KG-1, we obtained three distinct hDMP1 cDNA clones (Materials and Methods). Sequence comparison of each unique clone to the hDMP1 genomic clone (accession no. RG227L24) allowed identification of the genomic organization of hDMP1. The analysis revealed a perfect match for each of the 18 exons with corresponding splice donor and acceptor sites found in the genomic BAC clone (Table 1). hDMP1 contains a large 11 kb first intron with the translational start site located in exon 3 (Fig. 1A). Further comparison of the two unique hDMP1 message sequences with the hDMP1 genomic sequence revealed inserts of 172 and 211 nucleotides (nt), respectively, at nt 985 (accession no. AF084530). These sequences are identical to the genomic sequence of intron 9 and were generated by alternative splicing using two putative splicing acceptor sites as seen in Table 1. Based on our findings we now propose to refer to hDMP1 as hDMP1a and the new splice variants as hDMP1b and c respectively (accession no. AF202144 and AF202145). The open reading frames of hDMP1a and b encode identical initial amino acid (aa) sequences to hDMP1c up to the splice site at aa 237. However, after aa 237 hDMP1a and b show novel stretches of 35 and 48 aa, respectively, followed by a premature TAA stop signal occurring in the alternatively spliced intronic sequence (Fig. 1B). The newly defined hDMP1 isoforms still contain the complete acidic N-terminal transactivation domain (TAD), the cyclin D binding domain (CBS), a myb-homology remnant but no C-terminal TAD. The hDMP1a and b myb-homology remnant is composed of first 14/169 aa of the N-terminal sequence of the myb-homology domain followed by sequences derived from intron 9 and concluding with 35 aa showing homology to the C-terminus of the myb-homology domain (Fig. 1B).
contains additional 13 aa as compared to [], bridging the 14 and 35 aa remnants of the N- and C-terminal myb-homology region. The predicated length of proteins encoded by hDMP1[], [], and [] would be 760, 272 and 285 aa, respectively.

We next performed cell-free transcription and translation assays to verify the existence of protein translation from the hDMP1[] and [] message cDNA and to determine the approximate molecular weight of the translated proteins. HDMP1 expression plasmids produced all protein isoforms and displayed the molecular weights of 125, 31, and 32 kDa for hDMP1[], [], and [] respectively (Fig. 1C). These data provide evidence that two new human DMP1 splice variants can be expressed from cloned hDMP1[] and hDMP1[] message and both encode shorter proteins than found for full-length hDMP1[].

**Expression patterns of hDMP1 mRNA splice variants in primary hematopoietic cells**: hDMP1 is expressed in cells obtained from spleen, peripheral blood, and thymus (2). To investigate the expression patterns of the two novel hDMP1[] and [] mRNA splice variants and hDMP1[] in hematopoietic cells, we designed primers flanking the intronic insertions of hDMP1[] and [] allowing us to amplify all three variants in the same PCR reaction and thereby allowing intra-transcript comparisons (Fig. 2A). A number of distinct hematopoietic cell lineages were used as poly-A mRNA sources for evaluation of hDMP1 transcript expression. As can be seen in a representative panel of RT-PCR results presented in Fig. 2B, expression of hDMP1[], [] and [] was seen in RNA samples obtained from freshly isolated CD34+ hematopoietic cells (7/7 samples) derived from mobilized peripheral blood, peripheral blood derived mononuclear cells (PBMNCs) (6/6 samples), and cultured macrophages (MC) derived from peripheral blood (3/3 samples). In CD34+ cells the ratio of hDMP1[] to hDMP1[] transcripts was decreased (0.6±0.1;
n=5), indicating increased relative amounts of hDMP1\(a\) transcripts as compared to hDMP1\(b\). PBMNCs, a heterogeneous population of T-cells, B-cells and monocytes, expressed equivalent amounts of hDMP1\(a\) and \(b\) mRNA transcripts (hDMP1\(a\):hDMP1\(b\) ratio 0.9±0.1; n=6). In contrast to CD34\(^+\) cells, macrophages derived in tissue culture from peripheral blood monocytes predominantly and consistently demonstrated an increased hDMP1\(a\):hDMP1\(b\) ratio (3.3±1.0; n=3) implying increased relative hDMP1\(a\) transcript amounts as compared to hDMP1\(b\). In all cells populations examined from peripheral blood hDMP1\(g\) transcripts were weakly expressed.

To provide further insight into hDMP1 transcript expression in developing myeloid cells, cord blood CD34\(^+\) cells were differentiated in tissue culture using conditions that favored myeloid development. The presence of hDMP1 splice variants was assessed at various times early in differentiation and in culture derived neutrophils (Fig. 2C, top right panel) and monocytes (Fig. 2C, bottom right panel). Myeloid stages of cells grown in culture were confirmed by phenotypic and morphologic criteria. After one day of differentiation a marked down-regulation of hDMP1\(b\) was observed (Fig. 2C). During the entire differentiation period relative levels of hDMP1\(b\) stayed low whereas hDMP1\(a\) was the predominant splice variant identified. There were no substantial changes in hDMP1\(a\) transcript expression. In contrast, terminally differentiated neutrophils expressed relatively lower amounts of all hDMP1 splice variants, but increased amounts of hDMP1\(a\) vs. hDMP1\(b\), whereas monocytes expressed, relatively, higher amounts of hDMP1\(b\) transcripts. Assessment of hDMP1 transcripts from freshly isolated neutrophils obtained from blood confirmed (data not shown) what was seen in culture-derived neutrophils, the predominance of hDMP1\(a\) as compared to hDMP1\(b\) and lower amounts of total transcripts.
In summary, hDMP1 splice variants were expressed in all hematopoietic lineages evaluated. However, distinct patterns of hDMP1\(a\) and hDMP1\(b\) RNA transcript expression were found in freshly isolated CD34\(^+\) cord blood progenitor cells and PBMNCs when compared to differentiating myeloid cells. Moreover, monocytes, macrophages and neutrophils expressed increased relative hDMP1\(a\) RNA transcripts levels. In all cell lineages assessed hDMP1\(g\) transcripts were less abundant compared to the other splice variants and transcript levels did not appreciably change during myeloid differentiation. Recently it has been reported that mDmp1 is increased in cells that are not actively cycling (1,3). Interestingly, it was found that freshly isolated CD34\(^+\) cells, a cell population reported to be not actively cycling, have increased relative levels of hDMP1\(a\) as compared to hDMP1\(b\), whereas CD34\(^+\) cells undergoing myeloid differentiation and terminally differentiated, non-cycling macrophages and neutrophils demonstrated increased relative hDMP1\(a\) transcripts levels as compared to hDMP1\(b\).

*Expression patterns of hDMP1 mRNA splice variants in T cells obtained from peripheral blood-*

It has been reported that mDmp1 was strongly expressed in the mouse thymus, which is primarily comprised of activated, cycling and differentiating T cells (25). In contrast, T cells isolated from the peripheral blood are not highly activated or cycling and differing patterns of hDMP1 splice variants might be associated with non-activated vs. activated T cells and may provide additional information on correlative roles of hDMP1 splice variants. Interestingly, it has been shown that T-cell activation leads to CD13/APN cell surface expression (26,27) and CD13 activation has been reported, in part, to be possibly regulated by mDmp1 (6). To determine whether hDMP1 splice variant patterns and CD13/APN cell surface expression was associated with hDMP1\(a\) and hDMP1\(b\) transcript changes in T cells, we compared relative hDMP1
transcript levels in activated versus non-activated T-cells. Flow cytometry analysis was used to evaluate increases in CD69 expression, a positive control for T-cell activation, on freshly isolated PBMC and after 6 days of CD3/CD28 antibody activation. It has been shown previously that CD13/APN expression is increased after T-cell receptor mediated activation (26). As can be seen in Fig. 3 this increased CD13/APN surface expression is paralleled by a significant decrease in relative amounts of hDMP1[] transcripts, as compared to [], resulting in an increase in the hDMP1[]/[] ratio (Mann-Whitney U, P=0.0172). These data support an association between the appearance of CD13/APN cell surface expression and an increase in the hDMP1[]/[] ratio after T cell activation.

*Transactivation by hDMP1[] is repressed by hDMP1[] and to a lesser degree by hDMP1[].* The unique hDMP1[] and [] transcript patterns in activated T cells and developing hematopoietic cells revealed that various isoforms may have specific functions during development. This fact, in combination with increased CD13/APN cell surface expression and the increased hDMP1[]/[] ratio after T cell activation, prompted us to investigate a more mechanistic assessment of the role of hDMP1[], [], and [] isoforms in CD13/APN gene regulation. To this end hDMP1[], [], and [] expression plasmids were co-transfected with an artificial reporter plasmid (pGL2-BS2) into 293T cells. PGL2-BS2 contains eight concatemerized DMP1 (CCCGTATGT) binding sites upstream of a minimal TK promoter (1). Co-expression of pcDNA3.1-hDMP1[] and pGL2-BS2 containing plasmids in 293T significantly increased luciferase activity at levels 20-fold above that seen with the empty control plasmid (Fig. 4A). This is within the range of activation reported with mouse Dmp1 (1). In contrast, hDMP1[] and [] containing plasmids when co-transfected with pGL2-BS2 did not activate gene expression and interestingly hDMP1[]
decreased basal luciferase activity as compared to the control, non-hDMP1 containing plasmid (Fig. 4A). These findings suggest that unlike the hDMP1\(
\) isoform, both the hDMP1\(b\) and g isoforms may not function as transcriptional activators.

Given that hDMP1\(\alpha\) and \(\beta\) isoforms did not function as transcriptional activators, we next investigated whether their effect was transcriptionally neutral or repressive in the presence of the hDMP1\(\alpha\) isoform. This was assessed by co-transfection of constant amounts of the hDMP1\(\alpha\) plasmid with increasing amounts of either hDMP1\(b\) or hDMP1\(g\) containing plasmids. As can be seen in Fig. 4B upper panel, co-transfection of hDMP1\(\alpha\) plasmid resulted in a dose-dependent inhibition of hDMP1\(\alpha\)-induced transactivation. Interestingly, this effect was not seen when hDMP1\(\beta\) plasmid was co-transfected with hDMP1\(\alpha\) (Fig. 4B, lower panel) Thus, hDMP1\(\beta\) but not hDMP1\(\gamma\) has a dominant-negative effect on the transactivation potential of hDMP1\(\alpha\).

The myeloid and T cell CD13/APN promoter, pMyo, was evaluated (28) to further investigate the function of hDMP1\(\beta\) and \(\gamma\) as repressors of hDMP1\(\alpha\)-mediated activation. It has been reported that mDmp1 and c-Myb synergize to transactivate CD13/APN gene expression (6). The CD13/APN promoter reporter may provide a more physiological situation than the minimal DMP1 consensus binding site reporter by allowing interaction of additional transcription factors, such as Ets-1 and Ets-2 to regulate CD13/APN gene expression (6). As shown in Fig. 4C the strong dominant-negative effect of hDMP1\(\beta\) on the transactivation activity of hDMP1\(\alpha\) was evident. In contrast to the DMP1 consensus site reporter, modest inhibition by hDMP1\(\gamma\) was found. However, hDMP1\(\beta\) repression of hDMP1\(\alpha\) activity was less pronounced than that of hDMP1\(\gamma\) and was not dose-dependent. These results show using both an artificial, DMP1 consensus binding site and the myeloid/T cell CD13/APN promoter that both novel
isoforms do not transactivate and that hDMP1 does not significantly inhibit the transactivation activity of hDMP1.

**Stable U937 cell line transfectants of hDMP1 and hDMP1 show reduced CD13/APN levels**- To investigate the cellular effect of stable hDMP1 and expression on the regulation of cell surface expression of CD13/APN, hDMP1 and were ectopically expressed in the U937 cell line. This was accomplished using HIV vectors to deliver hDMP1 and genes to U937 cells line. The line was selected for studies based on the relatively high expression of CD13/APN on the cell surface. HIV vectors employed for cell delivery contained hDMP1 (VIPER) and hDMP1(VIPER) expression cassettes, a cellular internal ribosomal entry site (IRES) and the truncated nerve growth factor receptor (tNGFR) as a reporter to identify transduced cells through flow cytometry analysis. As a control the VIPER vector containing only the tNGFR gene was used. U937 cells were efficiently transduced with the selected VIPER vectors and then sorted so that all cells were ≥ 95% tNGFR-positive (data not shown). Analysis of CD13/APN expression in U937 cells transduced with VIPER showed reduced (Mann-Whitney U, P=0.0495; Fig. 5A) cell surface expression of CD13/APN (mean fluorescent intensity=15, n=3 independent evaluations) as compared to the VIPER expressing U937 cells (mean fluorescent intensity=41, n=3 independent evaluations). U937 cells transduced with VIPER did not show a statistically significant decrease in CD13/APN cell surface expression (mean fluorescent intensity=29, n=3 independent evaluations). To determine that transduced cells were expressing increased levels of hDMP1 or mRNA transcripts, RT-PCR analysis was performed using hDMP1 and specific primers. As can be seen in Fig. 5B U937 cell lines stably transduced with hDMP1 or vectors, expressed relatively, increased amounts of hDMP1 or as compared to mock-transduced U937 cells. That the loss of cell surface CD13/APN expression in cells ectopically expressing
hDMP1b is mediated via alterations in transcription can be directly seen in the results from CD13/APN RT-PCR analysis of cellular samples obtained from U937 stably expressing hDMP1b, hDMP1g or only the cellular tNGFR marker gene as a control. PCR products obtained at a different PCR-cycle number showed a decrease in CD13/APN transcripts in cells stably expressing hDMP1b, and to a lesser degree in hDMP1g as compared to the control. These results, when taken together with the CD13/APN promoter findings, are consistent with a repressive effect of hDMP1b and to a lesser extent hDMP1g on regulation of CD13/APN.

Decreased hDMP1b transcript levels in PMA-treated U937 cells and altered differentiation markers of stable U937 VIPERb cells upon PMA treatment- Consistent with findings from primary myeloid differentiation of CD34+ cells to monocytes and neutrophils (Fig. 2C), we have seen a significant decrease in relative amounts of hDMP1b transcripts upon PMA-induced differentiation of U937 cells to macrophages (Fig. 6A). In contrast, the hDMP1b transcript levels did not change. These findings suggested that a decrease in hDMP1b expression is associated with cytokine activation and differentiation.

To determine whether changes in the hDMP1a:hDMP1b RNA transcript ratio in favor of hDMP1b, i.e. increasing the cellular levels of the hDMP1b isoform, alter myeloid differentiation, U937 cells stably expressing hDMP1b (VIPERb) or only the control tNGFR reporter (VIPER) were differentiated to macrophages. Alterations in CD11c expression, cell morphology, and proliferation were quantified to evaluate the effects of hDMP1b on cellular function during differentiation. Differentiation of U937 cells to macrophages using PMA, is characterized by monocyte/macrophage specific gene expression (e.g. CD11c, CD11b) and characteristic morphological changes followed by loss of proliferation and terminal
differentiation (29). U937 VIPER\(b\) cells induced with PMA to differentiate to macrophages demonstrated reduced CD11c levels after 2 (40\%) and 4 days (18\%) as compared to the VIPER control cells (Fig 6B). Additionally, there were minor morphological alterations in nuclear to cytoplasmic ratio in PMA-induced U937 VIPER\(b\) cells as compared to VIPER control cells cultured under identical conditions. Only 88\% of the U937 VIPER\(b\) cells showed a nuclear to cytoplasmic ratio ranging from 1:2 to 1:4 as compared to 99\% of the U937 VIPER cells. Overall, these findings suggest that ectopic expression of hDMP1\(b\) altered some phenotypic and morphological markers associated with macrophage differentiation although adherent macrophages were nonetheless generated.

Continued proliferation of U937 VIPER\(b\) cells during PMA-induced macrophage differentiation-

Loss of cell proliferation is associated with terminal differentiation of U937 cells to macrophages. We observed that the pattern of cell proliferation during terminal differentiation to macrophages was altered in VIPER\(b\) cells, as compared to VIPER control cells (Fig. 7). U937 VIPER\(b\), in contrast to VIPER cells, maintained significantly higher proliferation when treated with 1, 3, or 10 nM PMA, a concentration range sufficient to induce macrophage differentiation in VIPER control cells. In summary, U937 VIPER cells treated with increasing PMA concentration showed a proliferation reduction of 9-72\% on day one and of 44-88\% on day two compared to VIPER\(b\) cells. On day two at the highest concentration of PMA used at least 95\% of the U937 VIPER and VIPER\(b\) cells were macrophages as judged by morphology. At day four, the two cell lines did not show significant differences in development (not shown).
These findings demonstrate that altering the hDMP1\(^a\):hDMP1\(^b\) RNA transcript ratio in favor of hDMP1\(^b\) modified the final step of macrophage maturation, which is cessation of proliferation.
DISCUSSION

The analysis of two unique hDMP1 message sequences isolated from myeloid cells revealed sequence identity corresponding to the use of intron 9 and further analysis indicate the presence of novel splice-acceptor sites in intron 9. These intronic insertions led to a frameshift and premature termination signals resulting in the truncation of full-length hDMP1 protein. The truncated hDMP1 and isoforms contain only a small segment of the myb-like homology domain and none of the C-terminal transactivation domain (Fig. 1B). Proteins corresponding to the correct size for the hDMP1 or open reading frame were identified by cell-free translation. Studies in mice have reported a single mouse Dmp1 message and differing molecular weights of mDmp1 proteins in the 125 kDa range which have been attributed to phosphorylation differences but 78 and 58 kDa protein species were also found. These shorter variants were only precipitated with antiserum against the N-terminus and may represent C-terminal truncated mDmp1 proteins (1). Investigations are currently underway address whether these truncated mDmp1 proteins may represent alternative splice variants similar to the ones found in humans. Splicing is an important means for the generation of protein diversity from a single gene in eukaryotes. It has been shown that the alternative selection of exon sequences during splicing is often regulated in a tissue-specific manner, possibly through distal promoter regulation (30,31). Increasing protein diversity from a single gene product has been shown to generate alternative forms of transcription factors, growth factor receptors, growth factors, and hormones. In regard to transcription factors, the presence of alternatively spliced transcription factors would increase the regulatory capacities for cell-specific gene expression. The finding of hDMP1 splice variants can be added to the growing list of identified alternatively spliced transcription factors such as Ikaros, AML-1, HIF-1, C/EBP, IPAS and Rel/NF-κB (32-37). Thus, the alternative hDMP1 and isoforms, as
compared to the hDMP1\(a\) isoform, may have potentially distinct functional properties in human hematopoietic cells.

Given the role of mDmp1 as a positive regulator of CD13/APN gene expression in hematopoietic cells, we first analyzed hDMP1 expression in CD34\(^+\) progenitor, myeloid and lymphoid cells. Bodner et al. (2) have previously reported that full-length \(hDMP1\) mRNA is highly expressed in brain and testis and expressed to a lower degree in peripheral blood leukocytes, whole spleen and thymus and is up-regulated in non-cycling cells (1,3). Our analysis of hDMP1 transcript patterns in CD34\(^+\) progenitor, myeloid and T cells identified a unique expression pattern for hDMP1\(b\) with the lowest hDMP1\(a/b\) ratios in non-cytokine activated CD34\(^+\) progenitor cells and freshly isolated peripheral blood mononuclear cells and highest hDMP1\(a/b\) ratios in mature myeloid cells. However, after cytokine activation of CD34\(^+\) progenitor cells or CD3/CD28 stimulation of T cells, hDMP1\(a\) was the predominantly expressed transcript. Our findings demonstrate that non-cycling cells such as macrophages and neutrophils express high hDMP1\(a/b\) ratios. Interestingly, CD34\(^+\) progenitor cells, a population reported to be non-cycling, display low hDMP1\(a/b\) ratios. These results when viewed together suggest that high hDMP1\(a/b\) ratios are associated with differentiation events. Overall, the findings in selected populations of hematopoietic cells suggest that expression of hDMP1\(a\) and \(b\) is activation and differentiation dependent.

It has been reported that the full-length mDmp1 is capable of transactivating the CD13/APN myeloid promoter (6); we now show that the alternative hDMP1\(a\) and \(b\) isoforms are unable to perform this function. Based on structural analogy to Inoue’s et al. (3) report on functional domain mapping of mouse Dmp1, the premature termination of the full-length hDMP1\(a\) resulting in hDMP1\(b\) and \(g\) would remove the majority of the myb-like binding region.
and the complete C-terminal transactivation domain (Fig. 1B). The remaining full-length domains present in hDMP1\(^b\) and \(^g\) isoforms include the N-terminal transactivation and cyclin D binding domains. The myb-like homology region remnants present in hDMP1\(^b\) and \(^g\) have lost the middle myb-like homology region (Fig. 1B), but remaining are the N-terminal most proximal 14 aa and a homology of 35 aa near the C-terminal end. The loss of the majority of the myb-like homology repeats and the conserved lysine at position 319, shown to be necessary for DNA binding, should preclude cognate DNA binding by the hDMP1\(^b\) and \(^g\) isoforms and thus abrogate reporter transactivation function (3). It has been shown that mouse Dmp1 mutants containing only the N-terminal transactivation and cyclin D binding domains do not function in transactivation assays with an optimized hDMP1-binding site reporter (3).

The aforementioned mouse studies are useful to provide a mechanistic explanation of the inability of hDMP1\(^b\) and \(^g\) to function as transactivators, however, the results presented herein suggest an active process of hDMP1\(^b\)-mediated repression. Although hDMP1\(^b\) was incapable of functioning as a transcriptional activator, co-transfection of hDMP1\(^b\) with hDMP1\(^a\) containing plasmids significantly repressed the transactivation function of hDMP1\(^a\) on the optimized hDMP1-binding site and CD13/APN-myeloid (pMyo) promoter reporters. The antagonist function of hDMP1\(^b\) on regulation of CD13/APN gene expression was further confirmed by the demonstration that ectopic expression of hDMP1\(^b\) significantly decreased CD13/APN message and cell surface expression in VIPER\(^b\) transduced U937 cells. Thus, hDMP1\(^b\) appears to be capable of functioning as a dominant-negative repressor. Although hDMP1\(^b\) altered the transactivation function potential of hDMP1\(^a\) in transient CD13/APN promoter evaluation assays and hDMP1\(^g\) ectopic expression decreased CD13/APN expression to some degree in U937 cells, it was not statistically significant as that mediated by ectopic expression of hDMP1\(^b\).
The decreased antagonist potential of hDMP1 as compared to hDMP1, may be related to the 13 additional amino acids from intron 9, as the result of alternative splicing, joining the N-terminal and C-terminal myb-like homology domain remnants. We speculate that hDMP1 may not play a major role in the regulation of hDMP1, given its extremely weak and non-changing expression in all cell types investigated as well as its low inhibition of hDMP1 function.

Why is there only partial abrogation of CD13 expression by ectopic hDMP1 expression in U937 cells? Studies by Inoue et al. dissecting the requirements of mDmp1 regulation of the CD13/APN promoter have implicated a cooperative interaction between c-Myb bound to its nominal site and Ets-1, Ets-2, or mDmp1 binding to specific GAA-cores 30 and 50 bases downstream (6). mDmp1 appears, at least in transient transactivation CD13/APN promoter studies in fibroblasts, to be superior to Ets factors for synergizing with c-Myb and increasing reporter gene activation. Nevertheless, Ets factors cooperate with c-Myb to activate CD13/APN gene transcription independent of mDmp1 (6). That Ets factors cooperate independently with c-Myb to regulate CD13/APN gene activation may help to place into perspective the finding of only partial reduction of CD13/APN expression in VIPER transduced myeloid cells ectopically expressing hDMP1.

The effect of ectopic expression of hDMP1 in U937 myeloid cells was not only limited to down regulating CD13/APN expression, it also altered expression of selected cellular markers associated with macrophage differentiation. The maintenance of proliferation in macrophages during terminal maturation, rather than cessation of proliferation which is concomitant with maturation, suggests two possibilities. The first is that ectopic hDMP1 expression disrupts the normal differentiation pathway required for halting proliferation during terminal maturation. The second possibility is that ectopic expression of hDMP1 overrides cellular control of
proliferation. It is known that mDmp1 is a positive regulator of p19<sup>ARF</sup> (also referred to as ARF or p14<sup>ARF</sup> in humans) expression and p19<sup>ARF</sup> is known to regulate the cell cycle. p19<sup>ARF</sup>, encoded from the Ink4a-Arf locus, is a nucleolar protein which activates the function of p53 through inhibition of hMDM2 to function as a tumor suppressor (4). It has been proposed that a major function of ARF may be to participate in the monitoring of the strength of mitogenic signals and prevent hyperproliferation of cells by prescribing alternative cell fates, such as p53-growth arrest or apoptosis (38). Recent studies also support the involvement of ARF in non-p53-mediated control of cell cycle pathways as well (39,40). In the context of possible p53-mediated cell cycle control in our findings of macrophage differentiation it has been reported that U937 cells have non-functional p53 (41), thus implying a non-p53 mediated effect. Studies on lymphoid cells and fibroblasts from DMP1-null mice demonstrate loss of cell cycle control and cellular hyperproliferation to selective signals (25). However, more studies are required to understand the role of DMP1 in normal hematopoietic development and if ectopic hDMP1 expression alters differentiation control and/or cell cycle regulators.

Truncated transcription factors generated by alternative splicing and lacking the transactivation and/or the DNA binding domains have been shown to function as dominant-negative repressors (42-47). How might hDMP1<sup>b</sup> and/or <sup>g</sup> impart their effects on hDMP1<sup>a</sup>? Since hDMP1<sup>b</sup> and/or <sup>g</sup> have deletions in the myb-like homology required for binding to cognate DNA, hDMP1<sup>a</sup> inhibition by hDMP1<sup>b</sup> and/or <sup>g</sup> may rely on interactions with unknown transcription elements or the formation of hDMP1<sup>b</sup>/<sup>a</sup> or hDMP1<sup>g</sup>/<sup>a</sup> heterodimers. It has been shown that D-type cyclins inhibit the ability of mDmp1 to transactivate the p19<sup>ARF</sup> promoter (5). There is some evidence from studies by Inoue and Sherr (3) that D-type cyclins do not function as mDmp1 corepressors, but may act via disruption of the mDmp1 transactivation complex rendering the
complex unable to bind DNA. It is possible, though speculative, that hDMP1\textsuperscript{b} or \textsuperscript{g} function in a similar fashion to inhibit the hDMP1\textsuperscript{a} transcription complex by interaction through the remaining domains. Alternatively, hDMP1\textsuperscript{b/g} may interact with hDMP1\textsuperscript{a} to form heterodimers, thereby removing or enabling hDMP1\textsuperscript{a} from the transcription complex. A concern, however, is the lack of obvious protein motifs in the N-terminal transactivation domain and the remaining regions that might predict protein heterodimer interaction. There are examples for both proposed mechanisms of transcriptional inhibition and studies are underway to determine the mode of action of hDMP1\textsuperscript{b} and \textsuperscript{g}.

In conclusion, we report herein the cloning of human hDMP1\textsuperscript{b} and \textsuperscript{g} and document unique hDMP1\textsuperscript{b} and \textsuperscript{g} expression patterns associated with activation and differentiation. HDMP1\textsuperscript{b}, and to some degree hDMP1\textsuperscript{g} represses hDMP1\textsuperscript{a} activity and ectopic expression of hDMP1\textsuperscript{b} reduces cell surface expression of CD13/APN. Regulation of hDMP1\textsuperscript{b} function may represent an additional cellular mechanism by which hDMP1\textsuperscript{a} activated genes, e.g. CD13/APN, an important player in myeloid differentiation and lymphocyte activation, are controlled. Ectopic expression of hDMP1\textsuperscript{b} leads to maintenance of proliferation in myeloid cells undergoing terminal maturation. These findings suggest that the ratio of hDMP1\textsuperscript{b}:hDMP1\textsuperscript{a} maybe critical for regulating proliferation at various stages of hematopoietic development. Additional studies are now needed to fully address the physiological significance of hDMP1 isoforms in p14\textsuperscript{ARF} and non-ARF regulated cell cycle pathways during hematopoietic development.
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FOOTNOTES

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1Abbreviations used are: aa, amino acid; cPPT, central polypurine tract; FITC, fluorescein; FL, Flt-3 ligand; PE, phycoerthrin; PMA, phorbol 12-myristate 13-acetate; h, human; hr, human recombinant; hrGCF, hr granulocyte colony stimulating factor; hrM-CSF, hr macrophage colony stimulating factor; hrSCF, hr stem cell factor; hrIL-3, hr Interleukin 3; hrIL-6, hr interleukin-6; IRES, internal ribosomal entry site; min, minutes; nt, nucleotide; RLA, relative luciferase activity; SAR, scaffold attachment region; tNGFR, truncated nerve growth factor receptor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. AF202144 and AF202145).
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    283, 682-686


FIGURE LEGENDS

FIG. 1. The hDMP1 splice variants use alternative intronic splice acceptor sites that produce different protein isoforms. A, The exon-intron structure of the hDMP1 gene and the alternative splicing that yield the new isoforms are shown. Full-length hDMP1[] contains 18 exons. HDMP1[,] and [] use alternative splice acceptor sites in intron 9 resulting in longer transcripts containing an additional 172 and 211 nucleotides, respectively. B, hDMP1 domains containing the transactivation domain (TAD), cyclin D binding sites (CBS), and myb-homology regions (MHR) were assigned by comparison to the well-characterized mDmp1 protein. HDMP1[,] and [] encode for a new common C-terminal end displaying 31% similarity to the C-terminal end of hDMP1[]’s MHR. The conserved functional domains with numbers showing amino acid positions, as well as the isoform-specific regions are indicated. C, In vitro transcription and translation of hDMP1[], [], and [] plasmid DNA. The open reading frame of hDMP1[], [], and [] encode for 760, 272 and 285 amino acids with the calculated molecular masses of 128, 31 and 32 kDa, respectively. Biotinylated lysine residues were incorporated during translation allowing detection with streptavidin-horseradish peroxidase after blotting of the SDS-PAGE gel. A plasmid encoding for luciferase (61 kDa) and an empty pcDNA3.1 expression plasmid were used as positive and negative control, respectively. A standard molecular weight marker is shown on the left.

FIG 2. mRNA expression patterns of hDMP1 splice variants in normal hematopoietic cells. A, The hDMP1 primers were designed in exon 9 and the exon 10/11 border in order to amplify all three hDMP1 splice variants as shown in the schematic representation. The intronic inserts in hDMP1[,] and [] will generate larger PCR products than hDMP1[] allowing identification of all
three variants in a single PCR reaction. The expected lengths of the PCR products are indicated. 

**B**, RT-PCR analysis of mRNA from peripheral blood CD34+ cells (CD34), peripheral blood mononuclear cells (PBMNC) and macrophages (MC) obtained from normal, healthy donors. The GAPDH gene product was used to determine whether an equal amount of RNA was amplified. hDMP1 and GAPDH PCR products were run on 2.5 and 1.5% agarose gels, respectively. **C**, Cord blood derived CD34+ cells were *in vitro* expanded and differentiated to neutrophils using hrG-CSF and to monocytes using hrM-CSF as described in Experimental Procedures. Cells were harvested at the days indicated and total RNA was extracted. RT-PCR analysis of hDMP1 splice variants was performed as described. Densities of the RT-PCR bands were measured using EagleEye software and \[\frac{a}{b}\] and \[\frac{c}{d}\] ratios were calculated. \[\frac{a}{b}\] ratios are displayed below the respective PCR bands, \[\frac{c}{d}\] ratios did not change. Representatives are shown from at least twice repeated experiments. GAPDH message expression levels served as controls for total mRNA integrity and to determine whether equal amounts of RNA were amplified.

**FIG 3.** hDMP1\[\frac{a}{b}\] down regulation parallels with increased CD13 expression in activated T-cells. The mean ± standard error of the ratio of hDMP1\[\frac{a}{b}\] to \[\frac{c}{d}\] transcripts is shown for lymphocytes untreated or activated with anti-CD3 and CD28 antibodies (*Mann-Whitney U, P<0.05, n=4 experiments). Peripheral blood T-cells were activated by culturing on CD3 and CD28 antibody coated tissue culture dishes for 6 days. To verify T-cell activation flow cytometry was used to assess the change in cell size (not shown) and increased expression of CD69 and CD13/APN [\% CD69+ or CD13+ cells]. RT-PCR analysis of hDMP1 splice variants and densitometric analysis was performed as described in Figure 2C. GAPDH transcript levels served as total mRNA controls.
FIG 4. hDMP1 isoforms are not equal in their transactivation potential on artificial DMP1 consensus binding site and full-length CD13/APN promoters. A, 293T cells were transiently transfected with 6 μg of pGL2-BS2 DMP1 consensus-site Firefly luciferase reporter plasmid, 20 ng of the Renilla luciferase expression plasmid pRL-TK, and 4 μg of the expression plasmid pcDNA3.1 containing no gene insert (control) or the gene for hDMP1[α], [β], or [γ]. Forty-eight hours after transfection, cells were assayed for luciferase activity. The promoter activity is reported as a ratio between Firefly luciferase and Renilla luciferase activity (relative luciferase activity, RLA). Results are given as the mean ± standard deviation of RLA from n=3 experiments. B, hDMP1[α], but not [γ] represses hDMP1[α] induced activation of a DMP1 consensus site reporter in a dose-dependent manner. 293T cells were transiently transfected with 1μg of the pGL2-BS2 Firefly luciferase reporter plasmid, 20 ng of the pRL-TK Renilla luciferase expression plasmid, 1.5 μg of pcDNA3.1-hDMP1[α] and varying amounts (0, 0.37, 0.75, 1.50, 3.00, and 6.00 μg) of the expression plasmid pcDNA3.1 containing the gene for either hDMP1[α] or [γ]. The pcDNA3.1 vector without insert was added where necessary to maintain equal amounts of transfecting DNA. Analysis was performed as described in A. C, Dose-dependent repression of hDMP1[α] induced human CD13/APN promoter activation by hDMP1[β], but not [γ] 293T cells were transiently transfected with 4.0 μg of CD13/APN Firefly luciferase reporter plasmid, 20 ng of the Renilla luciferase expression plasmid pRL-TK, 1.5 μg pcDNA3.1-hDMP1[β] and 0, 1.5, or 6.0 μg of the pcDNA3.1 plasmid containing either the hDMP1[β] or [γ] gene.
FIG 5. Altered CD13/APN cell surface expression in U937 cell lines ectopically expressing hDMP1 and g. A, Flow cytometry analysis of CD13/APN expression is shown for U937 cells stably transduced with the HIV vector VIPER containing only the tNGFR reporter (VIPER), or with VIPER vectors containing the genes for hDMP1 or g. The three transduced U937 cell groups were ≥95% tNGFR positive. Changes in CD13/APN expression levels on all three transduced cell populations were assessed by flow cytometry using CD13/APN-FITC antibody. Mean fluorescent intensity (MFI) of CD13/APN expression is indicated. Results are the MFI average (mean ± SD) of three independent experiments. U937 cells ectopically expressing hDMP1, but not g, show significantly reduced endogenous CD13/APN levels as compared to mock transduced cells (*Mann-Whitney U, P<0.05, n=3). B, RT-PCR of the transduced U937 cells. RT-PCR was performed using hDMP1 and g specific primers. Comparable, increased expression of the hDMP1 and g transgenes was detected in U937 cells transduced with VIPER and vectors, respectively, when taking into account endogenous levels of the mock transduced U937 cells. CD13/APN RT-PCR analysis from the same cell samples at 28, 30, and 32 cycles of amplification show decreased CD13/APN expression in hDMP1 and g transduced cells as compared to the parental line. Expression of the housekeeping control gene GAPDH is shown. Densities of the CD13/APN and GAPDH RT-PCR bands were measured and the CD13/GAPDH ratios are printed in the table.

FIG 6. Reduced hDMP1 transcript levels in PMA-treated U937 cells and decreased CD11c levels in stable U937 hDMP1 transfectants upon PMA-induced differentiation. A, Ratio of hDMP1 to g and h transcript expression during PMA induced macrophage differentiation of U937 cells. U937 cells were treated with 10 nM PMA for 48 hours and then total RNA was
extracted. PCR products were separated on a 2.5% agarose gel, and the density of the bands was measured using EagleEye software. The ratio of hDMP1\textsuperscript{a} to \textsuperscript{b} and \textsuperscript{a} to \textsuperscript{g} were calculated and the mean ± SD is presented (*Mann-Whitney U, P<0.05, n=3 experiments). ■ hDMP1\textsuperscript{a}/\textsuperscript{b} ratio, □ hDMP1\textsuperscript{a}/\textsuperscript{g} ratio. B, Reduced CD11c levels of PMA treated U937 VIPER\textsuperscript{b} as compared to VIPER cells. Median fluorescent intensities (MFI) were calculated from histograms of CD11c expression and are indicated. CD11c expression was measured at day 2 and 4. Non-PMA (MFI ≤ 5) and PMA (MFI ≥ 40) treated cells are shown in the same histogram. Cytospin preparations of U937 VIPER and VIPER\textsuperscript{b} cells, control or PMA-treated, were analyzed on day 4 using May-Gruenwald-Giemsa staining, confirming macrophage development of PMA-treated cells.

FIG 7. Continued proliferation of stable U937 hDMP1\textsuperscript{b} transfectants upon PMA-induced macrophage development. Proliferation during macrophage differentiation of the U937 VIPER and VIPER\textsuperscript{b} cell lines, expressing the tNGFR reporter only or the tNGFR reporter together with hDMP1\textsuperscript{b}, respectively. The cells were cultured in presence of 1, 3, or 10 nM PMA. Proliferation was determined by measuring incorporation of [\textsuperscript{3}H]-thymidine into the DNA. Results are given as counts per minute (cpm) of \textsuperscript{3}H-radiation from cell cultures set up in triplicate and the mean ± SD is presented (*Mann-Whitney U, P<0.05, n=3). A minimum of at least two experiments was performed. ■ U937 VIPER cells, □ U937 VIPER\textsuperscript{b} cells.
Table 1
Exon-intron boundaries of hDMP1. The positions of the exons are given according to the numbering of the hDMP1 cDNA sequence (GenBank accession no. AF084530). The splice acceptor and donor sites are shown in bold, the alternative 3’ splice acceptor sites producing exon 10 and are boxed.

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<th>5' splice donor</th>
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<td>cacag CTTCAG</td>
<td>TTCCG gtagg</td>
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<td>TACGC gtat</td>
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<td>2304 -2448</td>
<td>145</td>
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<td>AACAG gtact</td>
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<td>18</td>
<td>2449 -3767</td>
<td>1319</td>
<td>tacag ATCCC</td>
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</tbody>
</table>
Figure 1. Tschan et al.
A  hDMP1 mRNA species  RT-PCR products

<table>
<thead>
<tr>
<th>5'-primer</th>
<th>3'-primer</th>
<th>products</th>
</tr>
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<tr>
<td>hDMP1γ</td>
<td>8 9</td>
<td>211bp</td>
</tr>
<tr>
<td></td>
<td>10 11</td>
<td>450 bp</td>
</tr>
<tr>
<td>hDMP1β</td>
<td>8 9</td>
<td>171bp</td>
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<tr>
<td></td>
<td>10 11</td>
<td>420 bp</td>
</tr>
<tr>
<td>hDMP1α</td>
<td>8 9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>240 bp</td>
</tr>
</tbody>
</table>

B  CD34+247  CD34+245  PBMCN 01  PBMCN 02  PBMCN 04

α:β  0.7 0.6 0.6 1.5 3.0 1.3 4.4 1.1 2.6

C  Day 0  Day 1  Day 2  Day 3  Day 9  Day 10

α:β  0.5 2.4 2.7 2.4 1.8 1.5

Figure 2. Tschan et al.
Figure 3. Tschan et al.

- **hDMP1 α/β ratio**
  - Unactivated: 0.0
  - Activated (CD3/CD28): 2.0

- **CD13**
  - Unactivated: 1.7±0.7%
  - Activated: 26.2±6.8%

- **CD69**
  - Unactivated: 0.7±0.6%
  - Activated: 27.1±8.4%
Figure 4. Tschan et al.
Figure 5. Tschan et al.
Figure 6. Tschan et al.
Figure 7. Tschan et al.
Alternative splicing of the human cyclin D-binding Myb-like protein (hDMP1) yields a truncated protein Isoform that alters macrophage differentiation patterns
Mario P. Tschan, Kimberlee M. Fischer, Vivian S. Fung, Farzaneh Pirnia, Markus M. Borner, Martin F. Fey, Andreas Tobler and Bruce E. Torbett

J. Biol. Chem. published online August 12, 2003

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