PUBLISHED VERSION

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http://hdl.handle.net/2440/103301

Molecular Cloning of cDNA Encoding a Novel Platelet-Endothelial Cell Tetra-Span Antigen, PETA-3

By Stephen Fitter, Tim J. Tetaz, Michael C. Berndt, and Leonie K. Ashman

Platelet-endothelial cell tetra-span antigen (PETA-3) was originally identified as a novel human platelet surface glyco-protein, gp27, which was detected by a monoclonal antibody (MoAb), 14A2.H1. Although this glycoprotein is present in low abundance on the platelet surface, MoAb 14A2.H1 stimulates platelet aggregation and mediator release. We now report isolation of a cDNA clone encoding PETA-3 from a library derived from the megakaryoblastic leukemia cell line MO7e. The clone encodes an open reading frame of 253 amino acids that displays 25% to 30% amino acid sequence identity with several members of the newly defined Tetraspan, or Transmembrane 4 superfamily. These proteins consist of four conserved putative transmembrane domains with a large divergent extracellular loop between the third and fourth membrane-spanning regions. PETA-3 has a single consensus sequence for N-linked glycosylation located in this extracellular loop. A single PETA-3 RNA transcript (1.6 kb) was detected in RNA isolated from MO7e cells, bone marrow stromal cells, the C11 endothelial cell line, and several myeloid leukemia cell lines. No transcript was detected in the lymphoblastoid cell lines MOLT-4 and BALM-1. This pattern correlates well with previous protein expression data. Northern blot analysis of RNA from a range of human tissues indicated that the transcript was present in most tissues, the notable exception being brain.
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MONOCLONAL ANTIBODIES (MoAbs) have been used extensively to identify novel cell surface proteins and to investigate their functions. We previously described a murine IgG1 MoAb, 14A2.H1, which bound to a 27-kD glycoprotein (gp27) that is present in human platelet and endothelial cell membranes, but is absent from the majority of leukocytes.[1] MoAb 14A2.H1 was studied in the Fourth and Fifth International Leukocyte Typing workshops and was found in both cases to be unique.[2-4] Of particular interest, this MoAb was shown to be capable of bringing about platelet aggregation and mediator release.[1] Recently we showed that MoAb 14A2.H1 synergizes with subthreshold doses of other agonists, ADP, adrenaline, collagen, and serotonin in mediating platelet activation.[5]

As with certain other MoAbs with platelet activating ability, for example CD9 MoAbs,[6] platelet aggregation induced by 14A2.H1 was shown to be dependent on binding by both the Fc region and the Fab region.[1,5] This implies that signalling may be mediated, at least in part, by FcγRII. Nevertheless, a MoAb is not capable of extensive cross-linking of FcγRII (as is aggregated IgG) and, therefore, the specific antigen may contribute to the signalling process. Consistent with this, F(ab')₂ fragments of a CD9 MoAb, together with goat antimouse Ig coupled to latex beads, were shown to bring about platelet aggregation and mediator release.[7]

We have now cloned the cDNA encoding gp27, and

shown it to be a novel member of the Transmembrane 4 superfamily (TM4SF), also known as Tetraspans.[8] This is a family of cell surface proteins characterized by four transmembrane spanning regions and includes other platelet and endothelial cell membrane proteins, namely CD9 and CD63. Hence, we have coined the name Platelet-Endothelial cell Tetra-span Antigen-3 (PETA-3) for gp27. While their precise functions are unknown, the TM4SF molecules appear to be part of multicomponent signalling complexes that affect a variety of cell adhesion, proliferation, and migration functions.

MATERIALS AND METHODS

Cell culture. The human myelomonocytic cell line RC-2a, the T-cell acute lymphocytic leukemia (T-ALL)–derived cell line Molt-4, the B-lymphoblastoid cell line BALM-1, the acute promyelocytic leukemia cell line HL-60, the erythroleukemia cell line HEL900, and African Green Monkey derived COS cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The human megakaryoblastic cell line MO7e was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and interleukin (IL)-3 (10 ng/mL). The murine fibroblastoid cell line L*tk⁻* was cultured in Ham's F12 medium supplemented with 10% FCS. The genomic transfectant T33.8[9] was cultured in Ham's F12 supplemented with 10% FCS and hypoxanthine-aminopterin-thymidine (HAT).

Purification and sequencing of gp27. Gp27 was purified from the membrane glycoprotein fraction of platelets as previously described.[1] Purified gp27 (10 to 20 μg) was electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. After staining with Coomassie Brilliant blue, destaining, and extensive washing with water, the 27-kD band was excised, dried, and digested in situ with 2 μg of modified trypsin (Promega, Madison, WI). After overnight incubation at 37°C, tryptic peptides were eluted from the gel and chromatographed on a microbore reversed-phase high performance liquid chromatography (HPLC) column (RP300, C8, 1.0 mm I.D. × 50 mm) as previously described,[10] except that the gradient was extended to 150 minutes and HCl (0.1%) used as the mobile phase modifier. Peaks were collected manually, reduced with β-mercaptoethanol in 0.1 mol/L NH₄HCO₃ (pH 8.0) and rechromatographed under similar conditions, except that the temperature was increased to 50°C, the gradient extended to 180 minutes, and trifluoroacetic acid (TFA, 0.1%) was used as the mobile phase modifier. Peaks collected from this second chromatographic separation were sequenced as previously described.[10]

Synthesis and cloning of reverse transcriptase-polymerase chain reaction products. First strand cDNA was synthesized from 1 μg

Table 1. gp27 Partial Peptide Sequence and Corresponding DNA Sequence

Sequence of gp27 tryptic fragments and extrapolated PCR amplification primers*

(y)	(h)																		(r)
		Q	P	G	H	E	A	V	T	S	A	V	(x)	Q	L	Q	Q	E	F
	5'	CAR CCN GGN CAY GAR GC	3'							3'	GTY RAN GTY GTY CTY AA								5'

Sequence of cloned RT-PCR products amplified from MO7e cDNA with degenerate primers†

pC1	5'	CAA CCT GGT CAT GAG GCT GTG ACC AGC GCT GTG GAC CAG CTT CAG CAG GAG TT	3'
pC2	5'	CAA CCT GGG CAT GAG GCT GTG ACC AGC GCT GTG GAC CAG CTG CAG CAG GAG TT	3'
pC3	5'	CAG CCA GGG CAT GAG GCT GTG ACC AGC GCT GTG GAC CAG CTC CAG CAA GAG TT	3'

* R = A + G; Y = T + C; B = C + G + T; N = A + G + C + T.

† RT-PCR products were generated and sequenced as described in Materials and Methods.

of MO7e poly (A$^+$) RNA using a First Strand cDNA Synthesis kit (Pharmacia, Piscataway, NJ). The buffer composition was adjusted to that recommended for optimal AmpliTaq activity (Perkin Elmer, Norwalk, CT) and 128-fold degenerate oligonucleotides, corresponding to the partial peptide sequence (Table 1), were added to a final concentration of 50 nmol/L. The volume was adjusted to 70 μL, and 2.5 U of AmpliTaq (Perkin Elmer) was added before thermal cycling (1 minute at 94°C, 2 minutes at 40°C, 2 minutes at 72°C) for 30 cycles. The oligonucleotide concentration was then increased to 10 μmol/L, and 3 μL of 10 × polymerase chain reaction (PCR) buffer (Perkin Elmer) and a further 2.5 U of AmpliTaq were added. The reaction volume was adjusted to 100 μL, and a further 30 amplification cycles were performed under the previous conditions. Amplified products corresponding to 53 bp were purified from a 6% acrylamide gel then cloned into a T vector constructed in pBluescript KS(+). Recombinant clones were identified, then sequenced according to the dideoxy method,[11] with T7 polymerase (Pharmacia) and a Superbase Reagent Sequencing Kit (Bresatec, Adelaide, Australia).

cDNA library construction and screening. Clone pC2 was used to screen a commercially prepared HL-60 cDNA library made in λZAP II vector (Stratagene, La Jolla, CA). Approximately 10^6 plaques from this library were screened using plaque lift hybridization.[12] After three cycles of plaque purification, a single positive clone was obtained. The pBluescript SK(−) from this clone was rescued according to instructions supplied by the manufacturer. The 800-bp insert from this clone was then used to screen a cDNA library constructed from 2 μgs of MO7e poly (A$^+$) RNA, in pBluescript KS(+) (Stratagene), using the Time Saver cDNA synthesis kit (Pharmacia) essentially as described by the manufacturer. Colonies corresponding to positive signals were retrieved and secondary and tertiary screenings performed. Positive clones were then subjected to nested deletion analysis using the Erase-a Base Exonuclease kit (Promega) and sequence determined for both strands. Deletion clones were sequenced with vector-specific T3 and T7 primers as described above.

RNA isolation and analysis. Poly (A+) RNA was isolated from cultured cells as described elsewhere.[13] A total of 1.5 μg of poly (A+) RNA and 10 μg of total RNA were fractionated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to Hybond N+ nylon membrane (Amersham, Arlington Heights, IL), cross-linked at 0.45 J/cm^2 (Hybaid cross-linker), then hybridized to probes labelled with [α-^{32}P]-dATP by random priming using the Klenow fragment of DNA polymerase I.[12] Commercially obtained human multiple tissue Poly (A+) RNA blots (Clontech) were used (see Fig 5C and D). Filters were prehybridized at 42°C in 50% formamide, 6× SSC, 5× Denhardts, 0.1% SDS, 10 mmol/L Hepes pH 7.2, 1 mmol/L EDTA pH 7.2, 2 mmol/L sodium pyrophosphate pH 7.2, 160 μg/mL heat-denatured salmon sperm DNA and 50 μg/mL tRNA for ≥2 hours. Filters were probed (24 hours) in fresh buffer with 10^6 cpm/mL of labelled probe. Stringency washes were performed in 0.1× SSC + 0.1% SDS at RT followed by two 30-minute washes at 60°C. The PETA-3 probe was the 1.45-kb EcoRI fragment

from pGP27.1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 780-bp fragment from pHcGAP (ATCC, Rockville, MD).

Transient transfection of COS cells. The 1.45-kb EcoRI fragment from pGP27.1 was subcloned into the eukaryotic expression vector pcDNA-1 (Invitrogen, San Diego, CA) in both sense and antisense orientations according to standard protocols.[12] COS cells from a confluent 75 cm^2 flask were harvested by incubation with 1 mmol/L EDTA for 5 minutes in 5% CO$_2$ at 37°C. Cells were washed three times in MTPBS (50 mmol/L NaCl, 16 mmol/L Na$_2$HPO$_4$, 4 mmol/L NaH$_2$PO$_4$, 2H$_2$O, pH 7.3), then resuspended in 800 μL of MTPBS. DNA (10 μg) was added, mixed by gentle pipetting, and the suspension was transferred to a 0.4-cm electroporation cuvette (Bio-Rad, Hercules, CA) on ice. After 10 minutes, the cell/DNA mix was electroporated at 500 μFd capacitance and 0.3 kV voltage using a Biorad Genepulser coupled to a capacitance extender. Electroporated cells were mixed with 1 mL of RPMI + 10% FCS, overlayed on 1 mL 100% FCS, and pelleted by centrifugation at 200g for 5 minutes. Lysed cells and FCS were aspirated and the transfected cells were then resuspended in 20 mL of medium and used to reseed a 75 cm^2 flask. After a 72-hour incubation (5% CO$_2$, 37°C), 5 × 10^4 cells were cytocentrifuged onto glass slides, fixed for 30 seconds in buffered formol acetone, and subjected to immunohistochemical staining by the alkaline phosphatase antialkaline phosphatase (APAAP) method using MoAb 14A2.H1, as previously described.[1]

RESULTS

Generation of PETA-3 cDNA probe. Immunoaffinity purified gp27 was subjected to trypsin digestion and two independent peptides were sequenced. Both fragments contained identical sequences (Table 1). The high degree of coding redundancy and the undefined amino acid in the central portion of the peptide precluded direct screening of a cDNA library using degenerate oligonucleotides. Instead, fully degenerate oligonucleotides, based on the partial amino acid sequence, were synthesized to allow PCR amplification across the peptide sequence generating a 53-bp fragment. RNA was isolated from the megakaryoblastic cell line MO7e, which expresses abundant PETA-3 (data not shown), and first strand cDNA was synthesized using oligo(dt)$_{12-18}$. PCR amplification was then performed using the degenerate primers (Table 1) and amplified products corresponding to 53 bp were cloned and sequenced. The sequence of the cloned products accurately predicted the intervening amino acids confirming the cloned products as corresponding to gp27 (Table 1). Differences between the cloned products corresponded to positions of degeneracy within the oligonucleotide primers. From the sequences, amino acid ''x'' was

```
TCGCCCCCGCAGCTGCCGCCGCCGCCAGGGCCCGGACTCGGACGCGTGGTAGCCCCAGG          59

ATGGGTGAGTTCAACGAGAAGAAGACAACATGTGGCACCGTTTGCCTCAAGTACCTGCTG         119
 M  G  E  F  N  E  K  K  T  T  C  G  T  V  C  L  K  Y  L  L            20

TTTACCTACAATTGCTGCTTCTGGCTGGCTGGCCTGGCTGTCATGGCAGTGGGCATCTGG        179
 F  T  Y  N  C  C  F  W  L  A  G  L  A  V  M  A  V  G  I  W            40

ACGCTGGCCCTCAAGAGTGACTACATCAGCCTGCTGGCCTCAGGCACCTACCTGGCCACA        239
 T  L  A  L  K  S  D  Y  I  S  L  L  A  S  G  T  Y  L  A  T            60

GCCTACATCCTGGTGGTGGCGGGCACTGTCGTCATGGTGACTGGGGTCTTGGGCTGCTGC        299
 A  Y  I  L  V  V  A  G  T  V  V  M  V  T  G  V  L  G  C  C            80

GCCACCTTCAAGGAGCGTCGGAACCTGCTGCGCCTGTACTTCATCCTGCTCCTCATCATC        359
 A  T  F  K  E  R  R  N  L  L  R  L  Y  F  I  L  L  L  I  I           100

TTTCTGCTGGAGATCATCGCTGGTATCCTCGCCTACGCCTACTACCAGCAGCTGAACACG        419
 F  L  L  E  I  I  A  G  I  L  A  Y  A  Y  Y  Q  Q  L  N  T           120

GAGCTCAAGGAGAACCTGAAGGACACCATGACCAAGCGCTACCACCAGCCGGGCCATGAG        479
 E  L  K  E  N  L  K  D  T  M  T  K  R  Y  H  Q  P  G  H  E           140

GCTGTGACCAGCGCTGTGGACCAGCTGCAGCAGGAGTTCCACTGCTGTGGCAGCAACAAC        539
 A  V  T  S  A  V  D  Q  L  Q  Q  E  F  H  C  C  G  S  N  N           160

TCACAGGACTGGCGAGACAGTGAGTGGATCCGCTCACAGGAGGCCGGTGGCCGTGTGGTC        599
 S  Q  D  W  R  D  S  E  W  I  R  S  Q  E  A  G  G  R  V  V           180

CCAGACAGCTGCTGCAAGACGGTGGTGGCTCTTTGTGGACAGCGAGACCATGCCTCCAAC        659
 P  D  S  C  C  K  T  V  V  A  L  C  G  Q  R  D  H  A  S  N           200

ATCTACAAGGTGGAGGGCGGCTGCATCACCAAGTTGGAGACCTTCATCCAGGAGCACCTG        719
 I  Y  K  V  E  G  G  C  I  T  K  L  E  T  F  I  Q  E  H  L           220

AGGGTCATTGGGGCTGTGGGGATCGGCATTGCCTGTGTGCAGGTCTTTGGCATGATCTTC        779
 R  V  I  G  A  V  G  I  G  I  A  C  V  Q  V  F  G  M  I  F           240

ACGTGCTGCCTGTACAGGAGTCTCAAGCTGGAGCACTACTGACCCTGCCTTGGGCCTTGC        839
 T  C  C  L  Y  R  S  L  K  L  E  H  Y  *                            254

TGCTGCTGCACCCAACTACTGAGCTGAGACCACTGAGTACCAGGGGCTGGGCTCCCTGAT        899
GACACCCACCCTGTGCCATCACCATAACCTCTGGGGACCCCAACCTCAGAGGCAGCTTCA        959
AGTGCCTTTTGCTGCGCACCAATGCCCAGCAGGGGAGGTGAGGGGGGCTGGCGGGGCGAA        1019
GTTTGGGGGGTGTTTTGTGGGGCTCCCCGGACATACTCTCTGCCTGGTGGTCAGATGCAG        1079
GTTGGAAGGGGCCTTGCTGAGTGGCGCAAGGCCGAGCGTTCCCAGCAGGGGGAGAAACCC        1139
TTCACACCCCAGGCCCTTCAGGAACTGGGGCTTTGCCTTGCAGCCACATGGCCCCATCCC        1199
AGTTGGGGAAGCCAGGTGAGCTCTGACCCTTGGGCCTGGGCCTCTGCCCCTCCCAACCCA        1259
GCCGTCGTCTCCCTCGACAGCGCCCCTGCTGTCTTCCCCACCGCAGTCACCACCACCCGA        1319
AATGCCACGTGGTCACTGTGCACTGCCCTGTTCATGTGCCTCTGCGGGGCAGGGCCTTCC        1379
TGGTTTTGTACACTGCTGTACCCAGATGCCTACAACCATCCCTGCCACATACAGGTGCTC        1439
AATAAACACTTGTAGAGCAAAAAAAAA                                          1466
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Fig 1. Nucleotide sequence and deduced amino acid sequence of PETA-3. The cDNA sequence is shown above the deduced amino acid sequence. Nucleotide residues are numbered above and amino acid residues below. The partial peptide sequence is underlined. A possible N-glycosylation site is indicated by a broken underline, while a possible polyadenylation sequence is double underlined.

determined to be aspartic acid, and this was confirmed on reanalysis of the sequencing HPLC profiles.

Isolation of PETA-3 cDNA clones. Clone pC2 (Table 1) was selected for library screening based on codon usage data.[14] Initially, 10^6 recombinant phage from a HL-60 cDNA library in λZAP II vector were screened. After three cycles of plaque purification, a single positive clone was obtained. The rescued plasmid contained an 800-bp insert, which was truncated at the 5' end (data not shown). Given the low representation of PETA-3 transcripts in this library, a MO7e cDNA library in pBluescript KS(+) was constructed and screened with the 800-bp HL-60 gp27 clone. From the primary screen, 20 clones were isolated, of which 10 were further purified following secondary and tertiary screenings. On *Eco*RI digestion, these clones liberated inserts ranging from 1 to 1.5 kb. The clone with the most 5' sequence was determined (pGP27.1) and further characterized.

Primary structure analysis of PETA-3. The cDNA sequence shown in Fig 1 is derived from two overlapping clones, pGP27.1, which spans bases 1 to 1443 and pGP27.2, which spans bases 137 to 1466. Translational analysis of the

sequence showed a single open reading frame of 253 amino acids with the partial amino acid sequence in frame at positions 134-153. The partial peptide sequence is flanked by an arginine residue at position 133, indicative of a trypsin recognition sequence. The deduced protein molecular weight of 28 kD is slightly larger than that observed on nonreduced SDS-polyacrylamide gel electrophoresis (PAGE) gels.[1] A potential asparagine-linked glycosylation site is located at residue 159. The 5' proximal ATG codon, at position 20, is in good context for initiation of translation.[15] The in frame TGA stop codon is followed by a 636-bp noncoding sequence containing a potential polyadenylation signal (ATTAAA), and a poly(A) tail. Hydrophobicity plot analysis[16] of the putative gp27 coding region (Fig 2) indicates four groupings of hydrophobic residues, three at the amino terminus and one at the carboxyl terminus. Each hydrophobic grouping contains ≥22 amino acids, sufficient to represent membrane spanning domains. The putative transmembrane domains I, II, and III (Fig 2) are separated by short hydrophilic regions, whereas domains III and IV are separated by a large, mostly hydrophilic, grouping. The amino terminus
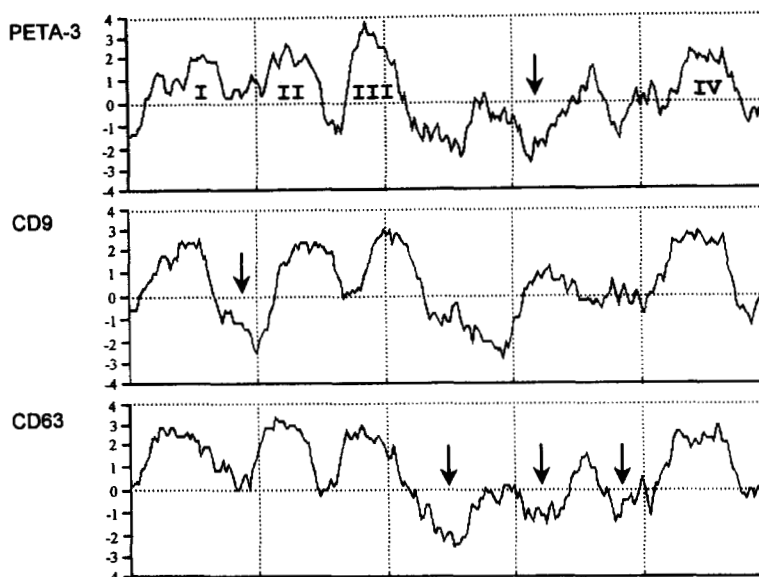
Fig 2. Hydrophobicity plot of the deduced amino acid sequence of PETA-3, CD9, and CD63. Plots were determined by the method described by Kyte and Doolittle.[16] Hydrophobic residues are positive. I-IV indicate putative transmembrane domains. Arrows indicate potential glycosylation sites.

consists of 16 mostly hydrophilic residues and the carboxyl terminus seven hydrophilic residues, suggesting either an intra or extracellular location for both ends of the molecule. No signal peptide consensus motif was found at the amino terminus, consistent with it remaining within the cytoplasm.

Homology to PETA-3. Sequence alignment and homology computations were performed at the National Centre for Biotechnology Information (NCBI, Bethesda, MD) by use of the BLAST Network service.[17] Results from these searches indicated that PETA-3 showed 25% to 30% amino acid sequence identity to several members of the newly defined transmembrane 4 (TM4SF) superfamily.[8] These molecules include CD9[18,19] (30% sequence identity), CD63[20] (29%), CD37[21] (25%), CD53[22] (29%), R2/C33 (CD82)[23] (28%), TAPA-1 (CD81)[24] (26%), CO-029[25] (27%), SM23[26] (23%), and A15[27] (29%). These molecules are structurally related and characterized by the presence of four putative transmembrane domains.[8] Comparison of hydrophobicity plots of PETA-3 with CD9 and CD63 (Fig 2) clearly demonstrates these proposed domains based on groupings of hydrophobic amino acids. An amino acid sequence alignment of PETA-3 with CD9 and CD63 (Fig 3) suggests conservation of the secondary structure by the presence of seven conserved cysteine residues. Assignment of domains based on hydropathicity of these molecules shows that homology is primarily found in the transmembrane domains. In particular, PETA-3 shows a high degree of sequence identity to CD9 in transmembrane domains I and IV (Fig 3). Several consensus motifs have been suggested for TM4SF molecules.[8,28] The CCG, PXSC, and EGC motifs (in the large extracellular domain), are clearly defined for PETA-3 at positions 156, 181, and 205 (Fig 1). The larger motif GCXAXXXEXXN (between TM domains 1 and 2), which corresponds to amino acids 78-88 (Fig 1), differed in two positions.

pGP27.1 encodes PETA-3. To confirm pGP27.1 as the construct encoding PETA-3, pGP27.1 cDNA was subcloned into the eukaryotic expression vector pcDNA-1 and a transient transfection was performed in African Green Monkey-derived COS cells (Fig 4). Expression of the antigen was detected by the APAAP staining technique using the anti-PETA-3 MoAb 14A2.H1. Antibody-dependent staining was observed in cells transfected with the sense construct (Fig 4B), but not the antisense construct (Fig 4A).

Northern blot analysis. Expression of PETA-3 mRNA was examined by Northern blot analysis of RNA isolated from various human cell lines, a murine L cell transfectant, bone marrow stroma, a spontaneous human umbilical vein endothelial cell (HUVEC) cell line, C11,[29] and adult human tissues. A single mRNA species (1.6 kb) was detected in RNA isolated from MO7e, HL-60, HEL900, and RC-2a human cell lines (Fig 5). No detectable signal was observed in RNA isolated from lymphoblastoid cell lines MOLT-4 and BALM-1, which correlates with previous protein expression data.[1] A single transcript, also of 1.6 kb, was observed in RNA isolated from the murine L cell transfectant T33.8 that was produced by human genomic DNA transfection and isolation with MoAb 14A2.H1.[9] This transcript was absent from nontransfected parent cells. A strong PETA-3 signal was observed in RNA isolated from TNFα-treated bone marrow stroma and the spontaneous HUVEC cell line C11. RNA isolated from C11 cells treated with cyclosporin A and/or TNFα did not appear to have altered levels of PETA-3 transcripts. A single RNA species of 1.6 kb was also observed in most human adult tissue (Fig 5C and D). High levels of PETA-3 transcript were observed in RNA isolated from heart, lung, pancreas, and prostate tissue. Low levels were observed in hematopoetic tissues including spleen, thymus, and peripheral blood leukocytes, and only negligible levels were detected in brain tissue.

DISCUSSION

We report here the molecular cloning of a cDNA encoding a new member of the TM4SF, PETA-3. The protein, previously termed gp27, was originally identified using the MoAb 14A2.H1, and the approach used here was to purify protein from a platelet glycoprotein extract, obtain partial amino acid sequence, and to use an oligonucleotide probe

```
                                   I
PETA-3    MGEFNEKKTTCGTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYISLLA------S    60
CD9          MPVKGGTKCIKYLLFGFNFIFWLAGIAVLAIGLW-LRFDSQTKSIFEQETNNNN         53
CD63         MAVEGGMKCVKFLLYVLLLAFCACAVGLIAVGVGAQLVLSQTIIQGATPGSLLP         53
homology        Gt C KyLLf  n  Fwlag av A G w l     S    s

                       II                              III
PETA-3    GTYLATAYILVVAGTVVMVTGVLGCCATFKERRNLLRLYFILLLIIFLLEIIAGILAYAY   115
CD9       SSFYTGVYILIGAGALMMLVGFLGCCGAVQESQCMLGLFFGFLLVIFAIEIAAAIWGYSH   113
CD63      V-------VIIAVGVFLFLVAFVGCCGACKENYCLMITFAIFLSLIMLVEVAAAIAGYVF   107
homology          yil  aG   m  g lGCC     E    l l f  Ll If  Ei A I  Y

PETA-3    YQQLNTELKENLKDTMTKRYHQPGHEAVTSAVDQLQQEFHCCGSNNSQDWRDSEWIRSQE   175
CD9       KDEVIKEVQEFYKDTYNKLKT--KDEPQRETLKAIHYALNCCGLAGGVEQF---------   161
CD63      RDKVMSEFNNNFRQQMENYP---KNNHTASILDRMQADFKCCGAANYTDWEKIPSMSKNR   163
homology        E  e  kdt  k       e                 CCG

                                                         IV
PETA-3    AGGRVVPDSCCKTVVALCGQRDHASNIYKVEGGCITKLETFIQEHLRVIGAVGIGIACVQ   235
CD9       -----ISDICPKKDVLETF----------TVKSCPDAIKEVFDNKFHIIGAVGIGIAVVM   206
CD63      -----VPDSCCINVTVGCG--INFNEKAIHKEGCVEKIGGWLRKNVLVVAAAALGIAFVE   217
homology        D C k  v                  c              igAvgiGIA V

PETA-3    VFGMIFTCCLYRSLKLEHY                                           253
CD9       IFGMIFSMILCCAIRRNREMV                                         226
CD63      VLGIVFACCLVKSIRSGYEVM                                         237
homology    fGmiF    L
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Fig 3. Amino acid sequence alignment of PETA-3, CD9, and CD63 antigens. The multiple sequence alignment was performed using MACAW by the method of Schuler et al.[52] Amino acids for all three molecules are numbered on the right. The lines above the sequence represent the putative transmembrane domains, which are labeled I-IV. Residues homologous to PETA-3 were determined; upper case represents residues conserved between all three molecules; lower case between PETA-3 and CD9; conserved cysteine residues are in bold type.

to screen a cDNA library from MO7e megakaryoblastic leukemia cells. Several lines of evidence indicate that the cloned cDNA encodes the antigen. Firstly, the deduced amino acid sequence includes the partial peptide sequence obtained from immunoaffinity purified gp27 (Fig 1). Secondly, a band corresponding to the native gp27 transcript was detected in a Northern blot of RNA isolated from a murine L cell subline, T33.8, expressing gp27 after transfection with human genomic DNA,[9] but not in untransfected cells (Fig 5). Finally, surface expression of the antigen was detected using MoAb 14A2.H1, following transfection of the cDNA into COS cells (Fig 4).
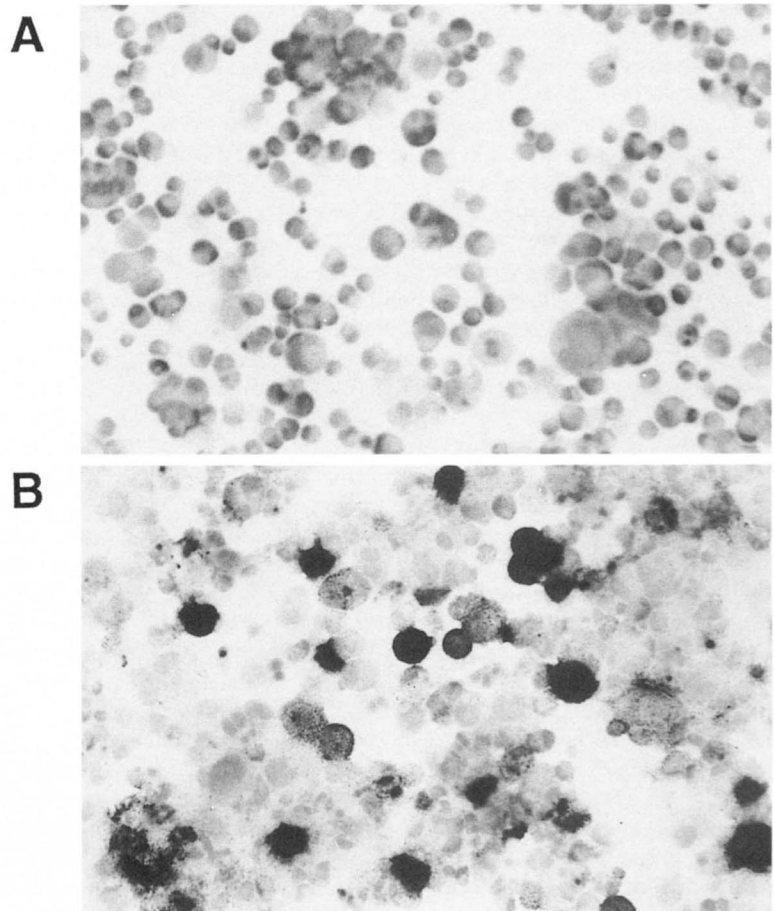
Fig 4. Detection of PETA-3 in transfected COS cells by APAAP. Cells were transfected with the pcDNA-1 vector containing PETA-3 cDNA in the antisense (A) or sense (B) orientation. Transfected cells were incubated with MoAb 14A2.H1 and three cycles of incubation with rabbit antibody to mouse immunoglobulin, then mouse monoclonal anti-alkaline phosphatase alkaline phosphatase soluble complexes. After incubation with Napthol AS-MX phosphate free acid substrate and Fast Red TR salt indicator, smears were counterstained with hematoxylin.
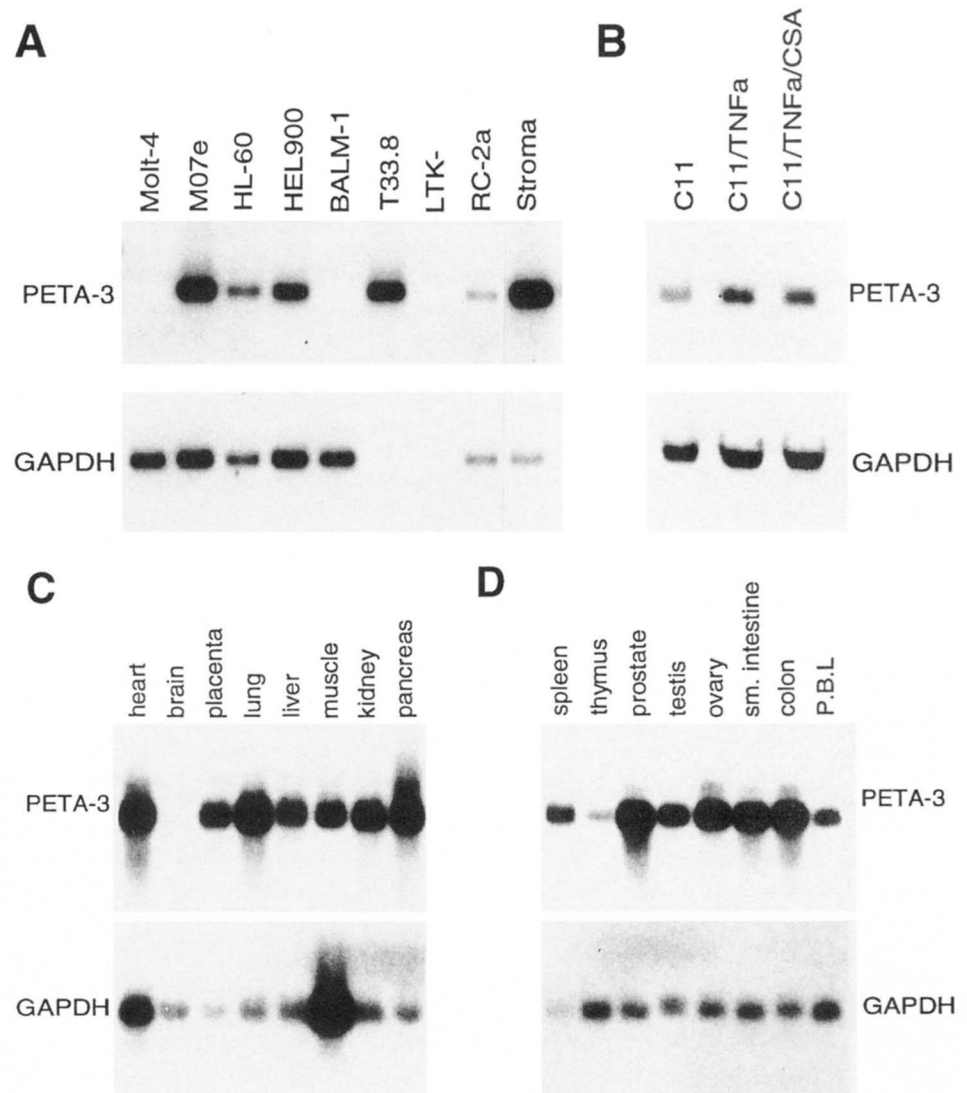
Fig 5. Expression of PETA-3 mRNA. Approximately 1.5 μgs poly (A+) RNA, isolated from eight different cell lines (A), 10 μgs of total RNA (B), and 2 μgs of poly (A+) RNA (C and D) was analyzed by Northern blotting. Blots were sequentially hybridized to PETA-3 (1.45-kb EcoRI fragment from pGP27.1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Where indicated (TNFa/CSA; TNFa) cells were incubated with cyclosporin A (1 μmol/L) and/or tumor necrosis factor α (100 U/mL), respectively.

Computer aided analysis of the cDNA clone showed a long open reading frame of 253 amino acids that encodes a core protein of molecular mass 28 kD. This is slightly larger than that observed on nonreduced SDS PAGE gels,[1] possibly due to the hydrophobic nature of the protein and/or the presence of several cysteine residues that may be involved in disulfide bonding. Amino acid sequence homology and strong secondary structure similarities inferred from hydrophobicity plots indicate that PETA-3 is a novel TM4SF[8] molecule. Hydropathicity analysis of TM4SF molecules suggests they span the membrane four times, indicative of Type III integral membrane proteins.[30] Membrane topology studies using CD81 (TAPA-1)[31] and epitope mapping experiments using the major hydrophilic domain of murine CD53[32] support this proposed structure and predict that both the amino and carboxyl termini lie inside the cell while the major hydrophilic domain of these molecules is extracellular. PETA-3 has a single putative N-linked glycosylation site located in this hydrophilic domain and is known to be glycosylated by virtue of its binding to concanavalin A-Sepharose.[1]

Using MoAb 14A2.H1, PETA-3 was originally identified as a novel platelet and endothelial cell antigen that has a restricted pattern of expression in peripheral blood and vasculature similar to that of other TM4SF members CD9 and CD63. CD63 is found in the dense granules of platelets[33] and the Weibel-Palade bodies of endothelial cells and is rapidly mobilized to the surface on activation.[34] Like CD9,[18] PETA-3 is constitutively expressed on the platelet surface, although at a much lower copy number, and displays a similar pattern of expression on microvascular endothelium in tonsil sections.[1] Other TM4SF members, CD81 and CD82, are also present on platelets and/or endothelium, but are predominately lymphoid markers, while the expression of the remaining family members on platelets and/or endothelium has not been reported.[8,35] Despite the restricted distribution of PETA-3 within the hematopoietic system (platelets and their precursors), Northern blot analysis presented here demonstrates that PETA-3 is widely and abundantly expressed in different tissues. A notable exception was brain tissue. Further analysis will be required to determine the

particular cell types that display this protein. We previously observed that epithelial cells, as well as endothelium, in tonsil were positive by APAAP, and have now shown that bone marrow stromal cells express PETA-3 mRNA at high levels. In the Vth International Workshop on Human Leucocyte Differentiation Antigens, MoAb 14A2.H1 was found to bind to a wide range of cells of stromal type.[35]

The biologic role of PETA-3 remains uncertain. While MoAb 14A2.H1 acts as a platelet agonist, this appears to depend, at least in part, on signalling via the immunoglobulin Fc receptor FcγRII.[1,5] Nevertheless, the low copy number of the protein on platelets (about 10^3/platelet; similar to FcγRII), led us to postulate that PETA-3 may form a complex with this receptor.[1] While there is no direct evidence for this proposal at present, other tetra-spans have been shown to be components of transmembrane signalling complexes. AD1, the rat homolog of CD63, has been shown to closely associate with the high affinity IgE receptor FcϵRI on basophils,[36] although the biologic significance of this association has not been determined. TAPA-1 (CD81) forms part of a signalling complex with CD21, CD19, and Leu-13 on B cells,[37,38] and with C33 and CD4 or CD8 on T cells.[39] CD53 is associated with CD2 on rat T cells and natural killer (NK) cells,[40] and with a complex of CD37, TAPA-1, CD82, and major histocompatibility complex (MHC) class I or II molecules in B cells.[41] It is interesting to note that these molecules can associate with different proteins in different cell types and that complexes often contain more than one member of the TM4SF. Homology between TM4SF members appears concentrated in the transmembrane domains. This has been suggested[25] to indicate a common effector function, whereas the divergence of sequences in the large extracellular domain may determine specificity for protein-protein interactions. Consistent with this, dissection of CD21/CD19/TAPA-1/Leu-13 complex on B cells using chimeric constructs showed that CD19 and TAPA-1 interact via their extracellular domains.[37]

TM4SF members have been implicated in a wide range of biologic processes. MoAbs directed against TAPA-1 and CD9 have been shown to cause homotypic adhesion of pre-B cells.[42,43] MoAbs directed against CD9 have also been shown to augment neutrophil adherence to endothelium[44] and to cause pre-B cell adherence to bone marrow fibroblast cells through induction or activation of fibronectin receptors.[45] CD63 has recently been shown to colocalize with von Willebrand factor (vWF) and P-selectin in Weibel-Palade bodies of endothelial cells,[34] implicating CD63 in adhesion processes. It has also been suggested, based on structural considerations, that TM4SF members may be components of ligand-gated ion channels,[8] although evidence is lacking.

Tetra-spans may also be involved in development and oncogenesis. CD9 was recently shown to be identical to Motility-Related Protein (MRP-1). Transfection of cell lines with cDNA encoding CD9/MRP-1 led to suppression of cell motility in vitro, and suppressed the metastatic potential of BL6 melanoma cells in vivo.[46] Furthermore, expression of this protein was recently shown to be inversely related to metastasis in human melanoma.[47] However, some TM4SF proteins appear to be associated with transformation. CO-029, L6, and ME491 (CD63) have all been identified as

tumor-associated antigens.[25,48,49] Recently SAS, a gene amplified in human sarcomas, was identified as a new member of the TM4SF.[50] PETA-3, like CD9, is expressed by some primary acute myeloid leukemia cells and cell lines, whereas these proteins are absent from normal myeloid progenitor cells, colony-forming unit–granulocyte macrophage (CFU-GM).[51] Whether these proteins play a role in leukemogenesis remains to be determined.

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1995 86: 1348-1355

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