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cDNA Expression Cloning of the 85-kDa Protein Overexpressed in Adriamycin-resistant Cells+

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Abstract: An 85-kDa cell surface protein recognized by monoclonal antibody MRK-20 was identified in adriamycin-resistant tumor cells. The expression of the 85-kDa protein has recently been reported to be associated with the differentiation of hematopoietic cells. However, the primary structure of the 85-kDa protein has not been determined. To clarify its primary structure, we carried out cDNA expression cloning of the 85-kDa protein with monoclonal antibody MRK-20. We found that the 85-kDa protein is identical to CD36 (GP VI), but at least two species of transcript exist in the tumor cells and one of these transcripts has a novel sequence at the 3'-region. The transcript with the novel sequence at the 3'-region was found to be expressed during the differentiation of hematopoietic cells.

The phenomena of multidrug-resistance is mainly caused by elevated expression levels of a cell surface protein, P-glycoprotein, which functions as an efflux pump and transports various antitumor agents outside the cells (1-6). In addition to the overexpression of P-glycoprotein in multidrug resistant K562/ADM cells, other biochemical changes including overexpression of an 85-kDa membrane protein also occur in the cell (7). The 85-kDa protein, recognized by monoclonal antibody MRK-20,

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Abbreviations used are: DMEM, Dulbecco's Modified Eagle's Medium; PBS, 0.01 M sodium phosphate (pH7.6)-0.15 M NaCl; EDTA, [Ethylenediamine]-tetraacetic acid; MOPS-Na, (3-[N-Morpholino]-propanesulfonic acid)-sodium salt; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate,

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was expressed in tumor cells resistant to anthracycline, mitoxantrone, etoposide and bleomycin, and in some normal cells including monocyte, granulocyte and endothelial cells (8-10). These observations suggest that the 85-kDa protein has physiological functions in various normal tissues as well as in some tumor cells which acquired resistance to adriamycin. Recently, we reported that the expression levels of the 85-kDa protein in hematopoietic cells were related to the stage of differentiation of the cells and that the 85-kDa protein had adhesive properties (11). The molecular function and the primary structure of the 85-kDa protein, however, are not known yet. In the present study, we successfully isolated cDNA of the 85-kDa protein by expression cloning which revealed that the 85-kDa protein is identical to CD36 (GP VI), an adhesive glycoprotein of platelets.

MATERIALS AND METHODS

Chemicals and restriction endonuclease. 12-O-tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals used in this study were analytical grade. Restriction endonuclease were obtained from Takara Shuzo Co., Kyoto, Japan.

Cell culture and induction of differentiation. Human myelogenous leukemia K562 cells and its adriamycin-resistant variant K562/ADM cells were cultured in RPMI 1640 medium (Nissui, Tokyo) supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY) (12). Human myelomonocytic leukemia THP-1 cells and human erythroleukemia HEL cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The THP-1 cells were treated with 15 nM TPA for 3 days to induce monocytic differentiation and the HEL cells were treated with 3 nM TPA for 5 days to induce megakaryocytic differentiation (11). COS cells were cultured in DMEM (Nissui, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum.

Isolation of RNAs. The total RNA from each cells was isolated with the guanidinium-thiocyanate / CsCl method, or with the acid-guanidinium-phenol-chloroform extraction method (13). Poly(A)$^+$RNA was obtained from total RNA by oligo d(T)-cellulose column chromatography.

Construction of cDNA libraries. Poly(A)$^+$RNA from K562/ADM cells was converted to cDNA using the cDNA synthesis kits (Bethesda Research Laboratories, Life Technology, Inc., Gaithersburg, MD). BstXI adaptors were attached and cDNA larger than 2 kbp length was separated by agarose gel electrophoresis. The size-selected cDNA was inserted into the BstXI site of the mammalian cell expression vector πH3M (14, 15) and 6 X 10^4 independent clones were obtained. A cDNA phage library of K562/ADM in λZAPII was constructed by ZAP-cDNA synthesis kits (Stratagene, La Jolla, CA) according to the instruction manual, and 5 X 10^5 independent clones were obtained.

Screening of cDNA libraries. The cDNA expression library in πH3M vector was introduced into COS cells by the DEAE-dextran method (14, 15). After 72 hours incubation at 37 °C, the transfected COS cells were detached from plates by incubation with PBS containing 5 mM EDTA / 0.02% azide. Cells were collected by centrifugation and resuspended in PBS containing 5% heat-inactivated fetal bovine

serum, 5 mM EDTA, and 0.02% azide. The cells were seeded on the panning plates which were coated with monoclonal antibody, MRK-20. These plates were incubated at room temperature for 3 hours and gently washed with PBS containing 5 mM EDTA / 0.02% azide. Plasmid DNA was recovered as a hirt lysate from the cells attached to the panning plates and used for transformation of E. coli MC1061/p3 (16). The transformed E. coli cells were used as an enrichment library for the next panning cycle. A cDNA phage library in λZAPII was screened by the standard plaque hybridization method with cDNA clone as a probe which was isolated by panning screening.

Sequence analysis of cDNA clones. A cDNA clone C-3 isolated by the expression screening was subcloned into pUC118 or pUC119. Other clones (13a, 14a, 32a, and 44a) which were isolated by plaque hybridization screening were rescued into pBluescriptSK(-). Subcloned cDNA were sequenced with the dideoxy method using the sequenase V 2.0 kit (United States Biochemical, Cleveland, OH) according to the instruction manual.

RNA blot analysis using cDNA fragments as a probe. Sample RNAs were electrophoresed through 1% agarose gel containing 2.2 M formaldehyde, 40 mM MOPS-Na (pH7.2), 0.5 mM EDTA and 5 mM sodium citrate and transfered to nylon filters (Nytran; Schleicher and Schuell GmbH, Dassel Germany). cDNA probes used in this experiment were 1) EcoRI-NdeI digested and 2) BlnI-XhoI digested DNA fragment of clone 13a cDNA, (see Fig. 2A). These probes were labeled with [α-^{32}p] dCTP (3000 Ci/mmol) using the Random Primed DNA Labeling kit (Boehringer Mannheim GmbH, Mannheim, W.-Germany) according to the instruction manual. Hybridization was performed at 65 °C in hybridization solution (6 X SSC/ 0.1% BSA/ 0.1% Ficoll-400/ 0.1% Polyvinylpyroridone/ 1% SDS/ 100 μg/ml denatured salmon testis DNA/ 100 μg/ml E.coli DNA/ 50 μg/ml polyA) for 15 hours and hybridized filters were washed in 2 X SSC/ 0.1% SDS at 50 °C. The blots were exposed to Kodak X-OMAT AR film at -80 °C for 96 hours.

RESULTS

Isolation of cDNAs encoding the 85-kDa protein. The cDNA library of K562/ADM cells was constructed in πH3M vector (14, 15), and transfected to COS cells by DEAE-dextran method. The putative plasmid clones encoding 85-kDa protein were enriched by "panning" procedures (17) on the MRK-20 coated plates. After three cycles of enrichment, each plasmid clone was independently transfected to COS cells and analyzed by indirect immunofluorescence with MRK-20. One of these clones, named C-3, encoded a cell surface protein recognized by the monoclonal antibody MRK-20 (Fig. 1). We rescreened a cDNA phage library with the C-3 clone as a probe in order to avoid possible errors, such as a mutation of plasmid DNA during enrichment cycle. After the rescreening, four independent clones, named 13a, 14a, 32a, 44a were obtained. Restriction enzyme mapping showed that these clones (13a, 14a, 32a, 44a, and C-3) contained 1.5~2.1 kbp long inserts and the inserts were classified into two groups according to the differences at the 3'-region (Fig. 2A). We used these clones for further sequence analysis.

Sequence analysis. Nucleotide sequence of the C-3 cDNA clone revealed a single open reading frame and the protein coding region was identical to the CD36

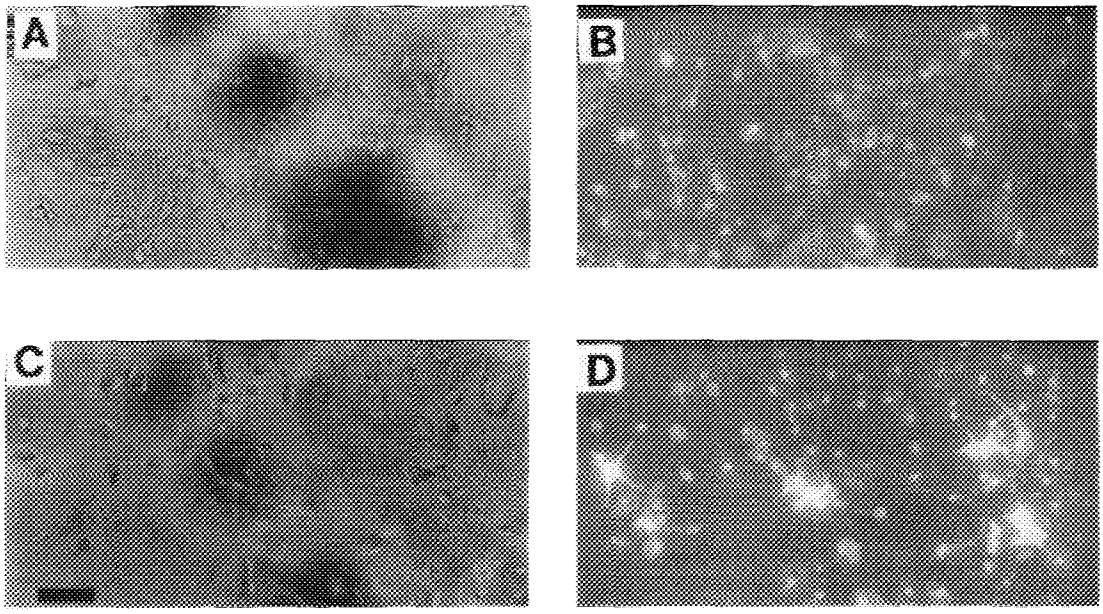
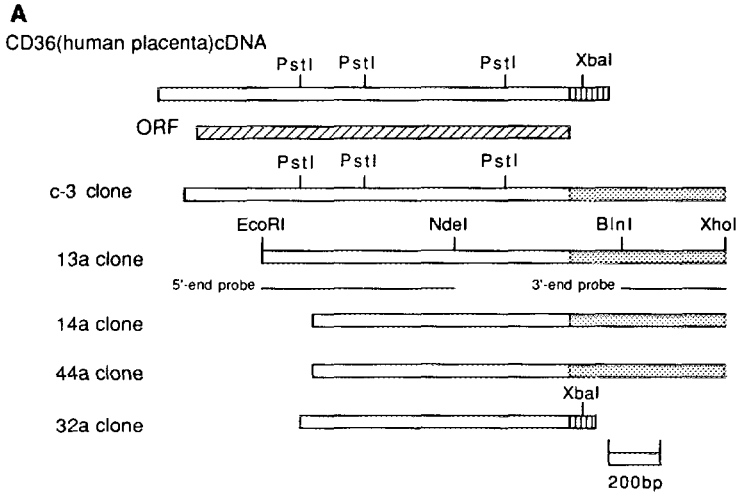


Fig.1. **Indirect immunofluorescence analysis of the transfected COS cells with MRK-20.** COS cells transfected with Mock plasmid DNA (A, B), and C-3 clone (C, D), by DEAE-dextran method were subjected to indirect immunofluorescence using MRK-20 as a first antibody, and anti-mouse IgG ($F(ab')_2$ fragment) conjugated with FITC as a second antibody. The cells were analysed by light (A, C) and fluorescence (B, D) microscopies. Bar: 100 μm.

cDNA(18). The sequence of the 3'-noncoding region, however, was different from that of CD36 cDNA reported previously (18) (Fig. 2B). Homology of the region from the stop codon to the 3'-end was about 43% between CD36 and C-3 cDNA. Our C-3 cDNA contains additional 374 nucleotides at the 3'-region. The other isolated cDNA clones were also sequenced. The sequences of clones, 13a, 14a, and 44a are identical with the sequence of the C-3 clone (Fig. 2A). On the other hand, clone 32a was identical to the CD36 cDNA except that the length of this clone was shorter by 87 nucleotides at the 3'-end and that 4 nucleotides (AAGT) were inserted at two nucleotides downstream of the stop codon (Fig. 2B). These sequencing data indicate that at least two different transcripts of the 85-kDa protein are expressed in K562/ADM cells.

RNA blot analysis of the 85-kDa protein. As the cDNA sequence analysis showed that at least two different transcripts of the 85-kDa protein exist, we carried out RNA blot analysis using two different cDNA probes (Fig. 2A). By using the EcoRI-NdeI DNA fragment from 13a cDNA as a 5'-end probe, the transcripts were detected as a major signal at 2.7 kb and as a minor signal at 5.1 kb and 2.0 kb in both K562/ADM and K562 cells (Fig. 3B). All of these transcripts were overexpressed in K562/ADM cells approximately 10-fold compared to K562 cells. RNA blot was further



B

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C-3   TCGAAAACAATAAAATAAAC----CTGGCTCAAGCACAAACCAATTTGTGTTGTTCTGATT
CD36  TCGAAAACAATAAAATAAGT----ATGTACCAA------AAAATATTGCTTCAATAATATT
32a   TCGAAAACAATAAAATAAGTAAGTATGTACCAA------AAAATATTGCTTCAATAATATT

C-3   CAATAATTGGTTTCTGGGTGGCCAATTCAGAAGAAGAGTGTACATGCTCAACAAATCCTAG
CD36  AGCTTATATATTACTTGTTTTCACTTTATCAAAGAGAAGTTACATATTAGGCCATATATAT
32a   AGCTTATATATTACTTGTTTTCACTTTATCAAAGAGAAGTTACATATTAGGCCATATATAT

C-3   GCCCTG-CATTCCTGTCATCCTCATCCGGGGGAAACACCATCATCCCAGTAGCTGCCCTAT
CD36  TTCTAGACATGTCTAGC-CACTGATCATTTTTAAATATAGGTAAATAAACCTAT-AAATAT
32a   TTCTAGACATGTCTAGC-CACTGATCATTTTTAAATATAGGTAAATAAACCTAT-AAATAT

C-3   TCAACTGCAACAGTCTCCAGGACCATCAGT-ATACTGCATTTCATGTGCACCAAATAT-TT
CD36  TATCACGCAGATCACTAAAGTAT-ATCTTTAATTCTGGGAGAAATGAG-ATAAAAGATGTA
32a   TAAAAAAAAAAAAAAAAAAA
          (poly(A)tail)

C-3   TGAAAGACATTTATAAATAATTGGCTTATGACTCATATTTCTCTATGAATACCTTCATACA
CD36  CTTGTGACCATTGTA-ACAATAGCACAAAT

C-3   GCAGGTATAACTCTTTTCTTTATGGGCTTAAATATTTTGTCACTGATCCTGCAAATGGACA
      TCATTTTAGCACACTAGCGGTTTATATTTTAAGGACCTTCATTCTCTGTTCTGCACCTCTT
      CTGGAAATTGAGTAAATTTTGCTTTTTTTTTTTACTCAGTTGCAACTTACGCTTGGCATCT
      TCAGAATGCTTTTCTAGCATTAAGAGATGTAAATGATAAAGGAATTATTGTATGAAATATT
      ACAAAGCGTAGACTATGCATTGTTATTCATTATAATATTTTTTGCTGTCATAATCGCCTCA
      TAAAGACAGGTTTCAACCATTAAAATATGTTCTTCCTTAAAAAAAAAAAAAAAAAAAA
                                          (poly(A)tail)
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Fig. 2. (A) Restriction endonuclease map of the 85-kDa protein cDNA clones and CD36 cDNA. EcoRI and XhoI sites are in multicloning sites of the vectors. XbaI site of CD36 and 32a clone is absent in C-3 and the other three cDNA clones. EcoRI-NdeI and BlnI-XhoI DNA fragments indicated by bars were used as the 5'-end and 3'-end specific probes, respectively.
(B) Comparison of nucleotide sequences of the 3'-region of 85-kDa protein cDNA clones and CD36. cDNA clones 13a, 14a, and 44a were identical to C-3 cDNA clone whose protein coding region is identical to the sequence of CD36. Sequences of 3'-region of C-3, 32a, and CD36 cDNA are presented in the figure. TAA shows stop codon and ATTAAA of C-3 and AATAAA of 32a correspond to the polyadenylation signal sequences. AAGT shows the four nucleotides insertion in 32a cDNA.

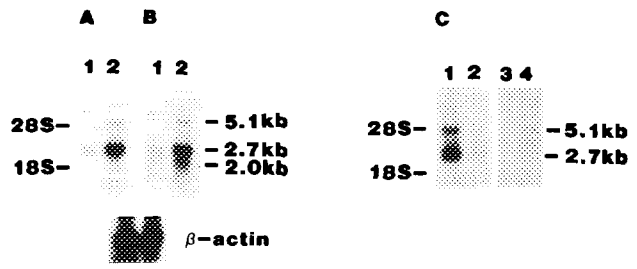

Fig. 3. RNA blot analysis of various cells using the 5'-end and 3'-end specific cDNA probes. (A,B) Each 5 μg / lane poly(A)+RNA from K562 (lane1) and K562/ADM (lane2) cells, and (C) each 10 μg / lane total RNA from HEL (lane1), differentiated HEL (lane2), THP-1 (lane3), differentiated THP-1 (lane4) cells were hybridized with 5'-end probe of EcoRI-NdeI DNA fragment (B), and with 3'-end probe of BlnI-XhoI DNA fragment (A, C) prepared from 13a cDNA clone. The blots were exposed at -80 °C for 96 hours.

carried out using BlnI-XhoI DNA fragment from 13a cDNA as a 3'-end probe (Fig. 3A). Transcripts were detected at 2.7 kb and 5.1 kb, but not at 2.0 kb. These results could indicate that 1) a 2.7 kb length major transcript corresponds to the C-3 type cDNA clone, 2) a 2.0 kb length minor transcript corresponds to the 32a clone, and 3) a 5.1 kb length minor transcript may be an immature transcript or cross-related gene. DNA blot analysis revealed that genomic amplification of 85-kDa protein DNA did not occur in K562/ADM cells (data not shown).

Previous report revealed that several hematopoietic cell lines expressed the 85-kDa protein during differentiation after TPA treatment (11). We also carried out RNA blot analysis after the TPA-induced differentiation of these cell lines using the 3'-end unique probe of 85-kDa protein (Fig. 3C). The expression level of 2.7 kb length mRNA transcript of the 85-kDa protein increased in THP-1 cells and decreased in HEL cells during differentiation by TPA treatment.

DISCUSSION

Previously, the expression of the 85-kDa protein in various tumor cells have been studied using monoclonal antibody MRK-20 (7-10) and it was speculated that the 85-kDa protein could be involved in the novel mechanism of adriamycin-resistance (atypical MDR). So far, the structure of the 85-kDa protein has not been elucidated. We carried out cDNA expression cloning employing monoclonal antibody MRK-20 directed against the 85-kDa protein and found that the 85-kDa protein is identical to CD36 (GP IV). CD36 is an adhesive cell surface glycoprotein which is shown to be a receptor for collagen and thrombospondin and it mediates the cytoadherence of *Plasmodium falciparum* parasitized erythrocytes (18-22). Recently we showed that the 85-kDa protein is a differentiation-associated antigen with adhesive property (11). Although the 85-kDa protein was found in various adriamycin-resistant tumor cells, we

recently found that the transfection of the 85-kDa protein cDNA tailed to confer drug resistance in receipient cells (Y. Sugimoto et al, submitted for publication). The possible involvement of this protein in the mechanism of adriamycin resistance is obscure at this moment.

Whereas the cDNA of CD36 was isolated from human placenta, our experiment revealed that a new type of cDNA with a novel sequence at the 3'-end is expressed in K562/ADM, HEL, and THP-1 cells. This new type cDNA corresponds to a 2.7 kb long mRNA transcript. Other 2.0 kb- and 5.1 kb- length transcripts were also detected in K562/ADM cells by RNA blot analysis. All these transcripts may be produced from the same gene because (1) all three species of transcript are overexpressed at equal ratios in K562/ADM cells as compared with K562 cells, (2) the difference between the 2.7 kb and 2.0 kb transcripts might be explained by this unique 3'-end sequences, including the different polyadenylation signal sequences, and (3) the 5.1 kb long transcript cross-hybridizes with both the 5'- and 3'-end cDNA probes of 85-kDa protein. The significance of the differences at the 3'-end sequences is not known, however, it could be proposed that the 3'-end sequences of mRNA could affect its stability or the efficiency of its translation into protein as reported previously (23, 24). Previous reports also found that several transcripts of CD36 were detected in C32 melanoma cells by RNA blot analysis (18) and the CD36 related glycoprotein, PAS IV was found in epithelial cells (25). It is possible that alternative splicings could occur from the same gene or that several closely related genes form a gene family.

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