Identification of Transferrin as a Progression Factor for ML-1 Human Myeloblastic Leukemia Cell Differentiation*

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We have previously demonstrated (Guan X.-P., Hromchak, R. A., and Bloch, A. (1989) Cancer Commun. 1, 111–115) that ML-1 human myeloblastic leukemia cells differentiate to monocyte/macrophage-like cells by the sequential action of competence and progression factors. Tumor necrosis factor-α, transforming growth factor-β, and the phorbol ester tetradecanoylphorbol acetate were found to induce competence, whereas a 77-kDa glycoprotein (DF77) isolated from mitogen-stimulated human leukocyte-conditioned medium initiated progression. In this communication we show DF77 to be an isoform of human transferrin. Hemin or soluble iron complexes did not induce differentiation progression, suggesting that the participation of transferrin in ML-1 cell differentiation may not be related to its iron-carrying capacity.

We have demonstrated that ML-1 human myeloblastic leukemia cells can be induced to differentiate to monocyte/macrophage-like cells when TNF-α, TGF-β, or the phorbol ester TPA is added to RPMI 1640 medium containing fetal bovine serum (FBS) (1–5). In the absence of FBS, neither the cytokines nor the phorbol ester were capable of inducing differentiation, indicating that FBS contains additional factor(s) required for differentiation to proceed. We showed subsequently (1) that the cytokines or the phorbol ester must be applied prior to or simultaneously with FBS in order to induce differentiation, characterizing these agents as differentiation competence factors. In contrast to FBS, mitogen-stimulated human leukocyte-conditioned medium (CM) was capable of inducing differentiation in the absence of added cytokines, indicating that CM contains both differentiation competence and progression factors (5). Antibodies specific for TNF-α and TGF-β inhibited CM-induced differentiation, demonstrating that they are the competence factors present in human CM. We consequently searched for the differentiation progression factor contained in CM and have identified it to be transferrin (Tf).

EXPERIMENTAL PROCEDURES

Materials—Human dipherr transferrin, human apotransferrin, TPA, pokeweed mitogen, ferrous sulfate, goat anti-human transferrin antiserum, V8 protease (sequencing grade), NBT, p-nitrophenyl phosphate, and ferric citrate were obtained from Sigma. Recombinant human TNF-α (Eschericha coli-derived, 5 × 10^5 units/mg protein) and a TNF-specific antibody were provided by Asahi Chemical Industry Co. (Tokyo, Japan), and TGF-β and its antibody were supplied by Oncogen Inc. (Seattle, WA). Hemin chloride was purchased from Calbiochem. Polyvinylidene difluoride membranes were from Millipore (Bedford, MA), and N-glycoside was obtained from Genzyme (Boston, MA). Blue Sepharose CL-6B and Mono Q anion exchange columns were obtained from Pharmacia LKB Biotechnology, Inc. FBS was from GIBCO.

Cell Assays—Exponentially growing ML-1 cells, washed twice with serum-free RPMI 1640 medium, were suspended at 5 × 10^6 cells/ml in serum-free RPMI 1640 medium and incubated for 16 h at 37°C. The cells were washed again with serum-free medium and resuspended at 6 × 10^6 cells/ml in serum-free RPMI 1640 medium. 0.1-m aliquots, containing 6 × 10^6 cells, were added to the wells of Falcon 96 multiwell plates for assays involving DF77, which was limited in quantity. 1-ml aliquots were added to Falcon 24 multiwell plates for all other determinations. The components listed in Table 1 were added at the concentration indicated, and the plates incubated for 48 h at 37°C. Cell numbers were counted by hemacytometer, and differentiation was assessed by determining the increase in the number of cells with EA rosettes (F, receptors) as well as the increase in acid phosphatase activity and in nitro blue tetrazolium (NBT) reduction (5). Human myeloblastic leukemia cells with five or more attached bovine erythrocytes were counted as EA-positive. At least 200 cells were counted per determination. Acid phosphatase activity was assayed by measuring, at 410 nm, the amount of p-nitrophenol released from p-nitrophenyl phosphate. NBT-dye reduction was assessed by counting the number of cells containing formazan deposits in their cytoplasm. 200 cells were counted per sample.

Isolation of DF77—Human leukocytes, obtained by leukopheresis, were separated by centrifugation through Ficoll-Hypaque (1.077 g/ml). After centrifugation at 1500 rpm for 30 min, the mononuclear cells present at the interphase were collected, and the platelets were removed by washing with RPMI 1640 medium. 5 × 10^6 cells/ml were then incubated at 37°C for 48 h in RPMI 1640 medium containing 10 mg/ml of pokeweed mitogen. Cells and debris were removed by centrifugation for 15 min at 1500 rpm, and the supernatant solution was dialyzed against 20 mM Tris.HCl buffer. The concentrate was applied to a 2.5-cm Blue Sepharose CL-6B column pre-equilibrated with the Tris.HCl buffer, and the material eluted with a 0.2–1.5 M NaCl gradient at a flow rate of 15 ml/h, with 2-ml fractions being collected. The fractions were assayed for their ability to induce ML-1 cell differentiation and the active fraction further resolved by fast protein liquid chromatography using a Mono Q anion exchange column. The chromatographic peak containing differentiation-inducing activity was analyzed by discontinuous SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (6).

Briefly, the material was kept on ice for 30 min in Laemmli sample buffer devoid of reducing agents, loaded onto a 12.5% polyacrylamide gel (4% stacking gel), and electrophoresed for 3 h at a constant current of 30 mA. The gel was cut into contiguous 0.5-mm slices, and each slice electroeluted for 3 h at 50 mA into 1 × Laemmli running buffer. Electroeluates were digested for 24 h, with two changes of buffer, against 2.0 nm Tris-HCl (pH 8.8) containing 0.1% glycerol.
were digested with N-glycanase (N-glycosidase F) in the absence of phenanthroline. The amino acid sequence digestion with V8 protease mended by Genzyme, using SDS-polyacrylamide gel electrophoresis for comparing molecular mass before and after deglycosylation with the amount of factor that provides for a 10% increase in acid phosphatase activity.

Identification of DF77—Proteolytic maps were generated in situ using V8 protease from Staphylococcus aureus, and the resulting peptides separated on a 15% discontinuous SDS-polyacrylamide gel (7). The gels were transferred for 8 h at 40 V to polyvinylidene difluoride membranes in 10% MeOH, 1 × Laemmli running buffer. The membranes were stained with 0.1% Coomassie Blue in 10% MeOH, and destained in 45% MeOH, 10% acetic acid. N-terminal amino acid sequencing of the peptides selected was performed in the laboratory of Dr. Paul Matsudaira at the Whitehead Institute for Biomedical Research (Boston, MA) as previously described (9).

Isolelectric point (pI) determinations were performed by two-dimensional polyacrylamide gel electrophoresis as previously described by O’Farrell (9). DF77 was subjected to isoelectric focusing in the first dimension (pH range 3-10) and to separation according to molecular weight in the second dimension, using SDS-polyacrylamide gel electrophoresis (4% stacking, 10% separating gel). The pI was determined by equilibrating consecutive 0.5-cm gel slices in 0.5 ml of distilled H2O overnight, followed by measuring the pH of each slice. Second dimension gels were silver stained to visualize protein spots. The carbohydrate content of DF77 was determined as recombinant by the procedures described under “Experimental Procedures.” Molecular weight in the second dimension, using SDS-polyacrylamide gel electrophoresis was previously recognized to exist (11). These include multiple glycosylation variants with pI values ranging from 6 to 7 (12) and a total carbohydrate content of approximately 4 kDa (13) (data not shown).

The close structural relationship between the two proteins was also reflected in their similarity of action. As shown in Table I (items 10–15), ML-1 cells were induced to differentiate when either DF77, apoTf, or FeTf was added to RPMI 1640 culture medium containing TNF or TPA (shown), or TGF-ß (not shown). Differentiation was measured by the increase in the number of EA-rosetting cells and by the elevation of acid phosphatase activity that occurs as the myeloblasts proceed towards the monocyte/macrophage stage. A third marker, nitro blue tetrazolium dye reduction, was used in place of acid phosphatase for confirming DF77-induced differentiation (Table I, items 10 and 11). The NBT assay requires a smaller number of cells and, as a consequence, a smaller amount of DF77, which was available in only limited quantities. ApoTf (items 5, 12, 13), at 1 µg/ml, equaled 10 units/ml DF77 (items 4, 10, and 11) in its ability to induce differentiation, whereas FeTf (items 6, 14, and 15), at 0.1 µg/ml, was more effective for differentiation induction. Unlike FeTf, apoTf has a reduced affinity for Tfr (11), and this may account for the smaller differentiation-inducing capacity observed. In the presence of TNF or TPA, hemin or ferrous sulfate (items 16–18) were not capable of replacing Tf for differentiation induction. Ferric ammonium citrate (300 µM) also lacked differentiation-inducing activity (data not shown). These findings may imply that the role of Tf in inducing differentiation is separate from its ability to deliver Fe to the cells.

Since the addition of TNF or TPA to cultures of ML-1 cells in RPMI 1640 medium plus 10% FBS led to differentiation (Table I, items 19 and 20), Tf contained in FBS was indicated to act as the progression factor required for inducing the differentiation process. This assumption was confirmed by the finding that, in the presence of an anti-Tf antibody in the cultured medium (Table I, items 21 and 22), differentiation did not ensue.

**DISCUSSION**

The importance of Tf and of its receptor (Tfr) for the transport of iron required for cell growth has been universally recognized (14–23). A pronounced increase in the number of surface Tfr has been found to occur in growth-stimulated and in proliferating cells (24–30), but after differentiation

![Peptide maps of DF77 and of authentic transferrin.](image)

Peptide maps were generated by digesting DF77 (lanes B, D, and F) and commercially available Tf (lanes A, C, and E) with 0 (lanes A and B), 0.2 µg (lanes C and D), or 2.0 µg (lanes E and F) of V8 protease as described under “Experimental Procedures.” Molecular mass markers given in kDa are shown on the left of the figure. The *superscripts* 1 and 2 denote peptides containing N-terminal amino acid sequences. The amino acid sequences determined are given under “Results.”

**RESULTS**

Analysis of CM by the procedures described under “Experimental Procedures” yielded a 77-kDa protein (DF77) which, in the presence of TNF-α, TGF-β, or TPA, proved capable of replacing FBS for induction of ML-1 cell differentiation. Sequencing of the N-terminal region and of an internal fragment showed DF77 to possess extensive homology with human Tf. Fig. 1 shows the proteolysis maps generated when authentic Tf (lanes A, C, and E) or DF77 (lanes B, D, and F) were digested with 0 (lanes A and B), 0.2 (lanes C and D), or 2.0 µg (lanes E and F) of V8 protease. As shown in lanes A and B, Tf and DF77 had the same electrophoretic mobility. These proteins also yielded identical proteolysis maps after digestion with V8 protease (lanes C versus D and E versus F). The amino acid sequence Val-Pro-Asp-Lys-Thr-Val-Arg-Trp-X-Ala-Val-Ser-Glu-His-Glu corresponding to the N-terminal region of intact DF77 (lane B, *superscript* 1) was found to be identical to that previously established for human Tf (10). However, since DF77 was not alkylated prior to analysis, the cysteine residue found present in position 9 of Tf was not recovered. Of the two DF77 peptide fragments sequenced, one (lane D, *superscript* 1) yielded the same sequence as provided by intact DF77, indicating that this fragment derived from the N-terminal region. The second peptide (lane D, *superscript* 2) contained the sequence Leu-Leu-X-Leu-Glu-Asp-Gly-Thr-Arg-Lys-Pro-Val-Glu-Glu-Tyr-Ala-Asn-X-His-Leu-Arg, corresponding to amino acids 561–581 of human Tf (10). Due to the lack of prior alkylation, no cysteine peaks were recovered at positions 3 and 17 of the peptide. A glutamic acid residue was found to be present at position 12 of the DF77-derived fragment, in place of the glutamine residue previously identified to occupy this position in a human Tf preparation (10). This alteration may indicate that DF77 is one of the approximately 20 human isoforms of transferrin that have previously been recognized to exist (11).

In addition to sequence homology and identical proteolysis maps, other biochemical parameters were also found to support the identity between Tf and DF77. These include multiple glycosylation variants with pI values ranging from 6 to 7 (12) and a total carbohydrate content of approximately 4 kDa (13) (data not shown).

FIG. 1. Peptide maps of DF77 and of authentic transferrin.

Peptide maps were generated by digesting DF77 (lanes B, D, and F) and commercially available Tf (lanes A, C, and E) with 0 (lanes A and B), 0.2 µg (lanes C and D), or 2.0 µg (lanes E and F) of V8 protease as described under “Experimental Procedures.” Molecular mass markers given in kDa are shown on the left of the figure. The *superscripts* 1 and 2 denote peptides containing N-terminal amino acid sequences. The amino acid sequences determined are given under “Results.”
transferrin, Tfr levels were observed to decrease markedly (31–45). Given the recognition that Tf is essential for growth, the present demonstration that Tf is also essential for the differentiation of ML-1 cells is of considerable interest. ML-1 cell differentiation proceeds from the G1 phase of the cell cycle without the need for cell division (46), and its stimulation by Tf may therefore be unrelated to Fe transport. The finding that supplementation of the medium with iron salts of different oxidation states (including ferrous sulfate, ferric citrate, or hemin) did not induce differentiation supports this suggestion. Instead of being merely an iron transport protein, the isoform of Tf isolated here may function as a specific induction, Tfr levels were observed to decrease markedly (31–45). Given the recognition that Tf is essential for growth, the present demonstration that Tf is also essential for the differentiation of ML-1 cells is of considerable interest. 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