Expression of the Protein Tyrosine Phosphatase β2 Gene in Mouse Erythroleukemia Cells Induces Terminal Erythroid Differentiation*

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We have cloned cDNA for protein tyrosine phosphatase β2, which had been implicated in erythroid differentiation of mouse erythroleukemia cells. Expression of cDNA constructs, in which β2 cDNA is placed under the control of mouse metallothionein-I promoter, by ZnCl₂ converted a significant portion (20 to 38%) of the cells to erythroid-like cells, which is 25–50% of the erythroid differentiation efficiency observed by conventional erythroid-inducing agents. Furthermore, introduction and expression of altered protein tyrosine phosphatase β2 cDNA constructs designed to produce the enzyme lacking the phosphatase activity inhibited erythroid differentiation by 100–20%, depending upon the concentration of erythroid-inducing agents employed. These results strongly suggest that protein tyrosine phosphatase β2 is involved in triggering erythroid differentiation in mouse erythroleukemia cells.

Erythroleukemia cells have been the subject of extensive studies as one of the best models for terminal differentiation (1–6). Upon exposure to inducing agents, mouse erythroleukemia (MEL) cells are converted to cells that exhibit all the characteristics of erythroid cells, including the loss of growth proficiency. Previously, we presented experimental results suggesting that dephosphorylation of phosphorylated tyrosine moieties of specific cellular proteins is involved in the early stage of MEL cell differentiation, which includes induction of the differentiation by protein tyrosine kinase inhibitors (7–10), inhibition of differentiation by a specific inhibitor (Na₃VO₄) of protein tyrosine phosphatases (PTPases) (11), and lack of tyrosine dephosphorylation in differentiation-defective mutant cells (11). Furthermore, two intracellular protein factors implicated in MEL cell differentiation had characteristics of a tyrosine-phosphorylated protein and a PTPase, respectively (12).

In more recent studies, we found that transcripts of two new protein tyrosine phosphatases (PTPases) (11), and lack of tyrosine dephosphorylation in differentiation-defective mutant cells (11). Furthermore, two intracellular protein factors implicated in MEL cell differentiation had characteristics of a tyrosine-phosphorylated protein and a PTPase, respectively (12).

We also isolated a clone with full-length cDNA for RIP (14) and PTPβ2. Here we report cloning of a full-length cDNA for PTPβ2 and show that introduction and subsequent expression of the full-length PTPβ2 cDNA in MEL cells, but not RIP cDNA, convert a significant portion (20–38%) of the cells to erythroid-like cells. Dominant-negative experiments using altered PTPβ2 cDNA constructs further suggested that PTPβ2 is involved in triggering MEL cell erythroid differentiation.

MATERIALS AND METHODS

Cell Culture—MEL cells (745A) were cultured at 37 °C in a CO₂ (5%) incubator in minimal essential medium with 12% (v/v) fetal calf serum. For colony formation, exponentially grown cells were incubated for 2 days under various conditions and plated on the medium containing methylcellulose (1.5%). Colonies were counted after 9 days of incubation as described previously (9).

Cloning of PTPβ2 cDNA—A cDNA library was constructed from MEL cells that had been incubated with 280 μM DMSO for 24 h (14), and clones were screened using a 377-base pair PTPase catalytic domain (BET) probe (15) radiolabeled with [32P]dCTP (111 TBq/mmol; ICN) (14). Of 1.5 × 10⁶ plaques, 30 plaques hybridized with the probe, and a clone (37–1) that contained the longest cDNA insert (~8 kbp) was subcloned. The nucleotide sequence was determined by the dyeodeoxy chain-termination method using Sequenase sequencing kit (U. S. Biochemical Corp.) and Tq polymerase on the Perkin-Elmer 373A automated DNA sequencer. We also isolated a clone with ~6 kbp of cDNA (45–10), and the DNA was sequenced.

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Plasmid Construction—The SacI/NotI fragments (6.0 and 7.6 kbp), which represent full-length PTPβ2 cDNA, were excised from clone (45–10) and clone (37–1), respectively. The DNA was blunt-ended and inserted into the blunt-ended Xhol site of pBMGneo vector (16). The resulting plasmids, termed pMgG2β-6 K (for 6-kbp cDNA) and pMgG2β-8 K (for 7.6-kbp cDNA) contain either the full-length 6- or 7.6-kbp PTPβ2 cDNA between the second intron of rabbit β-globin gene and its poly(A) signal, and their expressions are under the control of mouse metallothionein-I (mMT-I) promoter. The plasmids also carry neomycin resistance gene (neo). The blunt-ended Xhol fragment for full-length RIP cDNA (8 kbp) (14) was also inserted into the blunt-ended Xhol site of this vector, and the resulting plasmid was termed pMGRIP.

For pGK2Cys/Ser, a point mutation that should lead to the substitution of cysteine 1140 to serine was introduced into the full-length PTPβ2 cDNA in pBlueScript SK by Chameleon mutagenesis kit (Stratagene). The DNA was also appended at proline 52 with 10 amino acids of the human c-myc epitope (EQKLISEEDL). The SacI/NotI fragment (4.5 kbp) of this mutant PTP2 cDNA (β2Cys/Ser) was blunt-ended and inserted into the blunt-ended Ps1 site of pGK vector, including mouse phosphoglycerate kinase promoter and its poly(A) signal (17).

For pGK2β2AP, the NotI/blunt-ended BstXI fragment (3.0 kbp) of PTP2 cDNA was subcloned into the NotI/blunt-ended Xhol site of pBlueScript SK. This deleted fragment of PTP2 cDNA was appended with an artificial stop codon at threonine 1040. The SacI/SalI fragment (3.0 kbp) derived from this plasmid was blunt-ended and inserted into the blunt-ended PstI site of pGK vector.

Transfection—The plasmids (pMgG2β-6 K, pMgG2β-8 K, pMGRIP, pGK2Cys/Ser, and pGK2β2AP) were introduced into MEL cells using Lipofectin (Life Technologies, Inc.). In the case of pGK2β2Cys/Ser and pGK2β2AP, pGKneo vector was cotransfected with them. After 2 days,
Erythroid Differentiation Induced by PTP\(\beta\)

**FIG. 1. Deduced total amino acid sequence of PTP\(\beta\).** Amino acid sequence of PTP\(\beta\) deduced from the 7.6-kbp PTP\(\beta\) cDNA clone (37–1) is shown. Predicted signal peptide and transmembrane domain are underlined in bold. Putative fibronectin type III-like repeats are also underlined. The open box is the segment homologous to the catalytic domain of PTPases.

![Diagram](image)

**RESULTS**

Cloning and Characterization of PTP\(\beta\) Gene—Following exposure to erythroid-inducing agents such as DMSO or hexamethylenedisilazanide (HMBA), levels of two novel PTPase transcripts in MEL cells are sharply increased at a very early stage of differentiation (up to 24 h), and level off thereafter (13, 14). We first isolated the full-length cDNA sequence for PTP\(\beta\) (15) and subsequently the full-length cDNA for RIP (14). Total RNAs from these clones (Fig. 2) exhibited a band corresponding to the PTP\(\beta\) transcript among G418-resistant clones (34 clones for the 6-kbp cDNA and 1 clone for the 7.6-kbp cDNA). In order to investigate the relationship between PTP\(\beta\) and RIP transcription and MEL cell terminal differentiation, we constructed cDNA templates with exogenous PTP\(\beta\) (22) and RIP (23) expression and MEL cell terminal differentiation. We used the 7.6-kbp PTP\(\beta\) cDNA template under control of the mMT-I promoter.

![Diagram](image)
cDNA construct was introduced, 7.6- as well as 6-kbp transcripts (probably the spliced product of the 7.6-kbp transcripts) were induced (Fig. 2A, lower panel). For unknown reasons, those transcripts were expressed at a considerably high level in β2-57 even without ZnCl₂. We also examined a clone (β2-20) in which PTPβ2cDNA (6 kb) was present in the cells, but no PTPβ2 transcripts were detected (Fig. 2A). We also constructed a composite DNA in which a full-length cDNA for RIP (14) was placed under the control of the mMT-I promoter, and over 1 day β3 transfectants in which RIP transcripts are induced by ZnCl₂ were subsequently obtained. A typical induction pattern, exhibited by one of the clones (RIP13–35), is shown in Fig. 2C.

Hemoglobin Accumulation and Induction of Other Erythroid Differentiation Markers by the Expression of PTPβ2 cDNA—We next examined whether induction of PTPβ2 (or RIP) transcripts by ZnCl₂ affects erythroid differentiation. As seen in Table I, a substantial portion of β2-37 and β2-49 cells, which exhibited induction of 6-kbp PTPβ2 transcripts by ZnCl₂, became positive to benzidine staining, a specific staining for hemoglobin (25). Essentially the same results were obtained with β2-57 cells where 7.6- as well as 6-kbp PTPβ2 transcripts were also induced (Table I). On the other hand, no increase of benzidine-positive cells by ZnCl₂ was observed in parental cells (745A) and the control cells (β2-20), in which 6-kbp transcripts were not induced. None of the clones, including RIP13–35, which exhibited induction of RIP transcripts by ZnCl₂, showed an appreciable degree of increase in the number of benzidine-positive cells (Table I). The induction of hemoglobin in the PTPβ2 transfectants was confirmed by Western blotting for globin (Fig. 3A) although we detected small amounts of hemoglobin in β2-37, β2-49, and β2-57 cells without ZnCl₂, which was probably derived from the leaked transcription products as shown in Fig. 2A.

Although the efficiency of erythroid differentiation (20–38%) induced by ZnCl₂ was less than that induced (70–80%) by conventional inducing agents, these results strongly suggest that the PTPβ2 transcripts induced by ZnCl₂ were responsible for the accumulation of hemoglobin. Since intrinsic 7.6-kbp transcripts remained at a level comparable with those of ZnCl₂-induced 6-kbp transcripts regardless of the presence or absence of ZnCl₂ (see Fig. 2A), it seems that PTPβ2 translated from 6-kbp transcripts is responsible for the accumulation of hemoglobin, and there may be a specific mechanism to modulate the splicing of the 7.6-kbp transcripts in MEL cell differentiation. The reason for the low level of hemoglobin accumulation in β2-57 cells without ZnCl₂, despite the significant expression of PTPβ2 transcripts, is not clear. Either more sophisticated mechanisms exist to produce active PTPβ2 protein from the 7.6-kbp transcripts in addition to the splicing described above or β2-57 cells had been altered to be clonable. Otherwise, the cells with such a high level of the 7.6-kbp transcripts would have been differentiated and would have become non-clonable even without ZnCl₂.

We also investigated whether transcripts for δ-aminolevulinate synthase (ALAS-E), another marker for MEL cell differentiation (26), are induced following ZnCl₂ treatment. Fig. 3B shows that the ALAS-E transcripts were also increased in these cells (β2-49 and β2-57) by ZnCl₂. A similar pattern of induction was also obtained with the transcripts of another MEL cell differentiation marker, glycophorin (data not shown).

It has been observed that differentiated MEL cells lose their growth proficiency, thus failing to form colonies (27, 28). We found that the cells subjected to ZnCl₂ treatment significantly lose the colony-forming ability in β2-37 (~23%), β2-49 (~28%), and β2-57 (~38%) cells but not in 745A (~3%) and β2-20 (~3%) cells (Fig. 4). It seems that induced expression of PTPβ2 gene caused the cells to lose their growth proficiency as observed in erythroid differentiation induced by erythroid-inducing agents.

Irreversibility of Erythroid Differentiation by the Expression of PTPβ2 cDNA and Effects of Specific Differentiation Inhibitors—We also examined whether hemoglobin accumulation in the PTPβ2 cDNA transfectants (β2-37, β2-49, and β2-57) induced by ZnCl₂ is irreversible as observed in MEL cell differentiation. For this, the cells were incubated with ZnCl₂ for 2 days, ZnCl₂ was removed, cell incubation was continued for another 3 days, and accumulation of hemoglobin was assayed. As seen in Table II, incubation with ZnCl₂ for 2 days was sufficient to induce hemoglobin accumulation, suggesting that
Effect of Altered PTPβ2 cDNA Expression on Erythroid Differentiation—To investigate whether PTPβ2 is actually involved in erythroid differentiation induced by erythroid-inducing agents, we constructed altered PTPβ2 cDNA and examined whether expression of the altered PTPβ2 cDNA affects erythroid differentiation. In one of the constructs (pGKβ2Cys/Ser), a cysteine residue in the PTPβ2 catalytic domain (position 1140) was replaced by serine, so that the PTPase should lose its catalytic activity while maintaining the total integrity of the protein (23). Another construct (pGKβ2AP) in which 595 base pairs of the 3'-PTPβ2 ORF cDNA was deleted should code for PTPβ2 lacking two-thirds of the C-terminal intracellular PTPase catalytic domain. These altered PTPβ2 cDNA constructs were placed under the control of a strong constitutive promoter, phosphoglycerate kinase promoter (17).

Among 53 transfectants, which were obtained after transfection with pGKβ2Cys/Ser, three clones (clones β2Cys/Ser-2, -8, and -20) exhibited significant levels of PTPβ2 transcripts that were apparently derived from the introduced altered cDNA constructs (Fig. 5A). Employing these cells, we examined whether erythroid differentiation induced by DMSO or HMBA is affected as a result of the constitutive expression of the altered PTPβ2 cDNA. Fig. 5B shows quite clearly that all the transfectants expressing the altered PTPβ2 cDNA (pGKβ2Cys/Ser) transcripts exhibited considerably lower levels of erythroid differentiation by DMSO (left panel) as well as HMBA (right panel) than those of the parental and control cells, which included transfectants without the altered cDNA expression (clones β2Cys/Ser-1 and -9). As often observed in such dominant-negative experiments, the inhibitory effect was most prominently seen at suboptimal concentrations of the inducers (the optimum concentrations for erythroid differentiation are 160 mM for DMSO and 5 mM for HMBA). The effect was also confirmed by Western blotting for hemoglobin accumulated in the cells (Fig. 5C). We obtained essentially the same results with all the transfectants (clones β2AP-2, -12, and -40) expressing PTPβ2AP transcripts that should be translated into protein molecules lacking a considerable portion of the cytoplasmic PTPase domain of the enzyme (data not shown). These results indicate that expression of altered PTPβ2 with no catalytic PTPase activity, either by amino acid substitution or deletion of the catalytic domain, inhibits erythroid differentiation induced by DMSO or HMBA, and the effect may be best explained as a result of competitive inhibition by altered PTPβ2 of normal PTPβ2 activity for a tyrosine phosphorylated substrates whose dephosphorylation is essential for erythroid differentiation.

DISCUSSION

We have demonstrated that expression of a specific PTPase, PTPβ2, induced biochemical and physiological changes that are quite similar to those associated with in vitro and in vivo erythroid differentiation. Those include: 1) induction of hemoglobin and ALAS-E and glycophorin transcripts, 2) loss of growth proficiency, 3) cellular commitment to erythroid cells, and 4) sensitivity to specific inhibitors for MEL cell erythroid differentiation. The dominant-negative experiments using transfectants expressing altered PTPβ2 transcripts further suggest that PTPβ2 is involved in in vitro erythroid differentiation induced by conventional erythroid-inducing agents.

The results presented above, however, are still premature to conclude that PTPβ2 is actually responsible for triggering erythroid differentiation. For example, although all the characteristics induced by the expression of PTPβ2 so far examined were essentially the same as that observed in erythroid differentiation, the maximum efficiency of differentiation (20–38%) by the expression of PTPβ2 was approximately half of those

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**TABLE II**

Cellular commitment and effect of inhibitors

| Treatment                          | Benzidine-positive cells (%) |
|------------------------------------|-----------------------------|
| **Parental cells (745A)**          |                             |
| DMSO                               | 0.0                         |
| DMSO (280 mM)                      | 43.2                        |
| DMSO (280 mM) + PMA (800 ng/ml)    | 12.2                        |
| DMSO (280 mM) + dexamethasone (40 μM) | 13.9                       |
| DMSO (280 mM) + Na3VO4 (50 μM)     | 15.6                        |
| **PTPβ2 transfectants**            |                             |
| −ZnCl2                             | 0.0                         |
| ZnCl2 (160 μM) + PMA (800 ng/ml)   | 27.0                        |
| ZnCl2 (160 μM) + dexamethasone (40 μM) | 10.0                        |
| ZnCl2 (160 μM) + Na3VO4 (50 μM)    | 0.3                         |

at 2 days (when PTPβ2 transcripts were fully induced), the cells had already entered into an irreversible stage in expressing a series of erythroid specific genes. This is essentially the same as so-called “commitment” in which exposure to erythroid inducing agents for one or two days is sufficient for MEL cells to enter the irreversible cascade of terminal differentiation (27, 28). Thus, it seems that the hemoglobin accumulation and other phenotypic changes observed following ZnCl2 treatment are not results of transient expression of genes, but closely associated with cellular commitment to terminal differentiation.

We also examined whether specific inhibitors for MEL cell terminal differentiation such as phorbol 12-myristate 13-acetate, dexamethasone, or sodium vanadate (Na3VO4) inhibit hemoglobin accumulation induced by ZnCl2. Table II shows that these inhibitors blocked the induction of hemoglobin accumulation by ZnCl2 as they do in erythroid differentiation induced by erythroid-inducing agents.

Thus, by all the criteria so far examined, the changes observed upon induction of PTPβ2 gene expression by ZnCl2 were, at least qualitatively, indistinguishable from those induced by erythroid-inducing agents.

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**FIG. 4.** Colony-forming ability of PTPβ2 transfectants after ZnCl2 treatment. The cells were cultured with DMSO (280 mM) or ZnCl2 (160 μM) for 2 days and plated on a semisolid medium (9). Colonies were counted after 9 days of incubation as described previously (9). Bars depict colony-forming ability (mean ± S.E., n = 8).

Parental cells (745A) were transfected with DMSO or ZnCl2 in the presence (or absence) of the inhibitors for 2 days and further incubated without them for 3 days. The cells were then stained with benzidine to score the benzidine-positive cells (25).
FIG. 5. Effect of the expression of altered PTPβ2 constructs (pGKβ2Cys/Ser) on erythroid differentiation. A, altered PTPβ2 transcripts in the transfectants. Transfectants (β2Cys/Ser-1, -9, -4, -8, and -20 for pGKβ2Cys/Ser) and parental MEL (745A) cells were cultured, and the cells (5 × 10^5) were collected at the exponentially grown phase. Total RNA (20 μg) were subjected to Northern blot analysis using 32P-labeled DNA for a PTPase catalytic domain (nucleotides 3,424–3,759) of PTPβ2 cDNA (BET) (15) as probes for detection of PTPβ2Cys/Ser transcripts. Arrows indicate the position of the transcripts in the transcript. B, appearance of benzidine-positive cells as a function of DMSO (left panel) and HMBA (right panel) concentrations.

(70–80%) obtained by conventional erythroid-inducing agents. Several possibilities can be considered to explain the difference. Expression, stability, or intracellular location of the PTPβ2 induced through the artificial cDNA constructs may be different from that induced by erythroid-inducing agents. Alternatively, another cascade of molecular events may exist besides the PTPβ2 dephosphorylation cascade, and activation of that or both cascades leads to the full induction.

There have been reports concerning expression of β-globin and other erythroid-specific genes by transcription factors (3, 29). Expression of transcription factors implicated for erythroid-specific gene expression, such as SCL and MafK, substantially increased the level of expression of β-globin (3, 29) and ALAS-E (3) transcripts in MEL cells as well as the number of benzidine-positive cells, which reached 10–20% (29) or 24–54% (3) of the total population. It is not clear, however, from these reports whether the expression of these transcription factors resulted in erythroid differentiation that accompanied growth arrest and cellular commitment, features specific to erythroid differentiation. As we reported previously, induction of PTPβ2 transcripts starts at a very early stage of differentiation, as early as 6 h after addition of inducing agents (13), whereas induction of these transcription factors starts at a later stage (24 h) of differentiation for SCL (29) or no induction at all occurs during differentiation for MafK (T. Yamamoto, personal communication). Along with the pleiotropic induction of erythroid-specific genes and phenotypes by the expression of PTPβ2, this involvement of PTPβ2 in erythroid differentiation, if any, should be in a step quite upstream of the differentiation cascade, well before it differentiates into more specific cascades including β-globin gene expression that occurs at later stages. If this is the case, one could speculate that PTPβ2, with a transmembrane domain in the molecule (Fig. 1), is a membrane-associated mediator of a signal from the cell surface, which is generated by erythroid-inducing agents, to nuclei where a series of transcription factors specific to erythroid genes are subsequently activated.

The molecular mechanism of how expression of a single specific PTPase (PTPβ2) converts MEL cells to the ones whose characteristics are very similar to erythroid cells is, of course, a matter of speculation at this stage of the research. Induction of PTPβ2 may dephosphorylate a specific phosphotyrosine-containing protein whose dephosphorylation triggers a molecular cascade for terminal differentiation. Alternatively, induced PTPβ2 may dephosphorylate a broad range of cellular proteins rather nonspecifically and that among the dephosphorylated proteins, there may be one (or more) specific protein that is responsible for erythroid differentiation. Western blot analysis of phosphotyrosine-containing cellular proteins indicated no significant changes in the patterns of major tyrosine-phosphorylated proteins following ZnCl₂ treatment (data not shown), suggesting that induction of PTPβ2 dephosphorylates only a limited number of specific proteins. Experiments to identify the target proteins using more sensitive detection procedures are currently underway.

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