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EXPRESSION AND MODULATION OF PROTEIN KINASE C ISOFORMS IN DIFFERENTIATION-COMPETENT AND DIFFERENTIATION-RESISTANT ERYTHROLEUKEMIC CELLS

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We have investigated eight of the protein kinase C (PKC) isoforms in Friend virus immortalized erythroleukemia cells. Using Western analysis, we detected the presence of the α, δ, ε, ζ and θ isoforms with no β, γ η. We compared levels and modulations of these isoforms among 2 lines with different susceptibilities to DMSO-induced differentiation, a cell line rendered differentiation resistant by constitutive expression of an exogenous c-*myb* gene and cell lines naturally resistant to differentiation. These comparisons have shown that DMSO-induced differentiation is associated with downregulation of the δ, ε and θ isoforms. High levels of c-*myb* expression prevent the downregulation of ε, but not δ and θ, and also appear to have resulted in lower constitutive levels of PKC α. These results help define which isoforms are important and provide clues as to what point in the signal transduction pathway they may be acting relative to another element, c-*myb*.
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Friend leukemia virus immortalized hematopoietic cells have provided a valuable model system for the study of erythroid differentiation. This is due to the property that some of these cell lines can be induced on treatment with DMSO to differentiate as assessed by hemoglobin synthesis. Using this system, Clarke, et al. (1) were first able to establish that the c-*myb* gene product is involved in this process. This was indicated by experiments in which an exogeneous c-*myb* gene was expressed at elevated levels in a differentiation-competent F-MEL cell line, rendering it differentiation-resistant. Previously, it had been reported that while DMSO alone induced differentiation, co-treatment of cells with TPA and DMSO prevented this differentiation, indicating a role for protein kinase C (PKC) in the process (2,3).

PKC is actually a family of proteins known as isoforms (α,β,γ,δ,ε,ζ,η,θ,ι,λ,μ) and it is the differential modulation of the activities of these isoforms which is thought to control

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differentiation, as well as a number of other cellular processes (4). In order to gain more insight into DMSO-induced erythroid differentiation regarding the involvement of PKC with emphasis on its interaction with c-*myb*, we have employed Western analysis to examine PKC isoform levels in several Friend-leukemia virus immortalized cell lines before and after DMSO treatment. We have compared these levels in differentiation-competent and differentiation-resistant lines. Among the differentiation-resistant cell lines are those originally resistant and a cell line rendered differentiation resistant by a constitutive c-*myb* gene.

MATERIALS AND METHODS

Cell culture. F-MEL cells can be induced to differentiate to an extent where 60-70% test positive for hemoglobin as assessed by benzidine staining. The BB88 cell line doesn't differentiate as completely as F-MEL exhibiting a 10-20% differentiation level. F-MEL-myb cells were derived from F-MEL cells by introduction of a constitutive c-*myb* expression vector rendering the line totally resistant to differentiation (1). The F-MEL and the F-MEL myb cell lines were generously provided by M. Clarke. The BB88, T27A, D2N, BC3A and BC16A cell lines, the latter four of which do not differentiate, were obtained from the American Type Culture Collection (ATCC). Extracts were obtained from cells untreated and treated with DMSO, TPA or both DMSO and TPA. DMSO treated cells were prepared by adjusting logarithimically growing cells to 1.6% DMSO for 5 days. TPA treatment was at 0.1 μg/ml.

Protein analysis. Cell pellets were lysed in Tris-Cl buffer containing 0.1% SDS and proteinase inhibitors. DNA was sheared to reduce viscosity, and protein concentration was determined with Bio-Rad protein assay dye reagent. 50 μg of total protein was mixed with an equal volume of 2X sample buffer (0.125 M Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue). Samples were heated in a boiling water bath for 5 min and then loaded onto a denaturing 6% polyacrylamide electrophoresis gel.

After separation, proteins were electrophoretically transferred to a nitrocellulose membrane. The filters were first treated with Tris-buffered saline (50 mM Tris-Cl [pH 7.5] 0.15 M NaCl) with 0.05% Tween 20 (TBST) containing 3% nonfat milk. This was followed by hybridization in 5 ml of TBST with 1% bovine serum albumin and an appropriate dilution antibody for 5 hr at room temperature. Anti c-*myb* antibody type I and anti PKC α antibody were from Upstate Biotechnology. Anti PKC δ, ε and θ antibodies were from Santa Cruz Biotechnology and anti PKC ζ was from Gibco. The blots were subsequently washed in TBST and then incubated with the appropriate immunoglobulin peroxidase-conjugated antibodies (Amersham) for 1 h. After washing in TBST, the blots were then developed using the enhanced chemiluminescence Western blotting (immunoblotting) system (Amersham).

RESULTS

Downregulation of c-myb mRNA levels on induced differentiation has been noted for F-MEL cells before (5,6). Figure 1 illustrates that $p75^{myb}$ was readily detected in uninduced cells and became undetectable on DMSO-induced differentiation. Extracts from BB88 cells, which don't differentiate as fully, showed less of a reduction. Because TPA treatment prevents this differentiation, we examined the effect of this reagent on $p75^{myb}$ levels. TPA treatment alone has no effect on $p75^{myb}$. As we have noted before (7), treatment of F-MEL cells with both TPA and

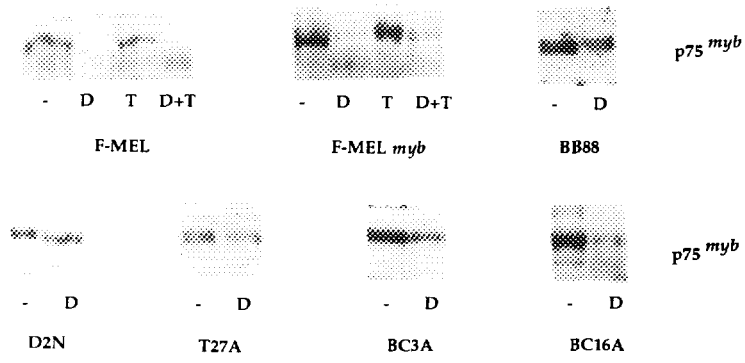

Fig. 1. Western analysis of $p75^{myb}$ expression in Friend-virus-immortalized cell lines before and after treatment with DMSO and or TPA. Lanes labeled — indicate no treatment; D indicates treatment with DMSO; T indicates treatment with TPA; D&T indicates treatment with both DMSO and TPA. Cell lines from which the extracts were obtained are as labeled.

DMSO surprisingly still resulted in loss of $p75^{myb}$ protein; even though the presence of TPA prevents the cells from differentiating. Thus, under these circumstances, downregulation alone of $p75^{myb}$ is not sufficient for induction of differentiation. This suggests that the function of PKC might lie at a point downstream of c-*myb* in the sequence of events leading to differentiate.

Examination of $p75^{myb}$ in F-MEL myb cells reveals that, as expected, an elevated level of $p75^{myb}$ has indeed been achieved. Unlike its F-MEL counterpart, DMSO treatment did not eliminate $p75^{myb}$ levels. Densitometric analysis suggests F-MEL myb cells express approximately 2.5 times more $p75^{myb}$ than F-MEL cells and that DMSO treatment of F-MEL myb cells resulted in an 83% reduction of $p75^{myb}$. This indicates that exogenously expressed $p75^{myb}$ is being downregulated along with the endogenous protein, which in turn indicates that protein or mRNA degradation is at least part of the mechanism of $p75^{myb}$ downregulation by DMSO. This would explain why we (7) and others have had trouble expressing $p75^{myb}$ at high enough levels to achieve inhibition of differentiation. High levels of $p75^{myb}$ have only been achieved by the use of a vector pMbMI/DHFR which co-expresses dihydrofolate reductase (1). Growth of transformants of this gene can be grown in successively higher concentrations of methotrexate leading to high copy numbers and higher expression of vector encoded genes. None of the lines resistant to induced differentiation were capable of completely downregulating $p75^{myb}$. Two lines, BC3A and BC16A showed some downregulation of $p75^{myb}$ levels while the D2N and T27A lines showed no change on DMSO treatment.

PKC α was expressed in all lines with no apparent modulation in levels between differentiated and undifferentiated cells. As seen in Fig. 2, a 75% reduction was noted in F-MEL*myb* cells compared to F-MEL cells suggesting that high levels of exogeneous c-*myb*

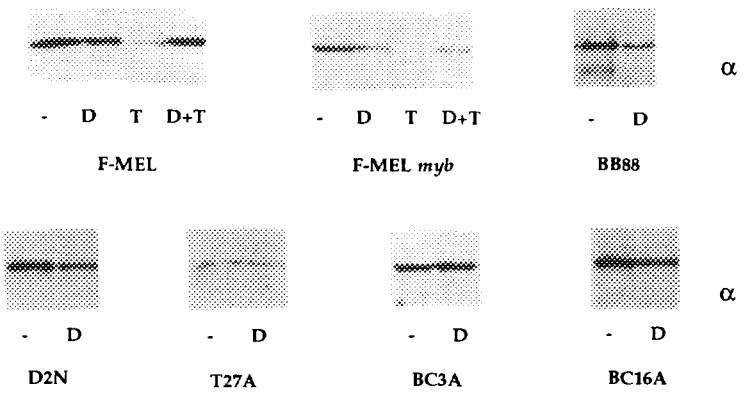

Fig. 2. Western analysis of PKC α expression in Friend-virus-immortalized cell lines. Labelling is as in Fig. 1.

expression have downregulated levels of PKC α and thus may be in part responsible for the cell's inability to differentiate. TPA-treatment of either lines resulted in a diminution of PKC α levels and surprisingly this effect was prevented by co-treatment with DMSO and TPA. These results suggest that if PKC α is involved in cellular differentiation, that absolute levels of PKC α may be more important in the F-MEL cell lines' ability to differentiate than modulation of levels during DMSO treatment.

Previous reports have associated PKC δ with the ability of erythroleukemia cell lines to differentiate. These studies compared PKC δ levels in uninduced differentiation-competent cell lines with different susceptibilities to differentiation. One report implies high levels are important (8), while two others imply low levels are critical (9,10). Our data showed more PKC δ in the line less susceptible to differentiation than F-MEL and even more in lines completely resistant (Fig. 3). When we examined the modulation of PKC δ, we found that DMSO-induced

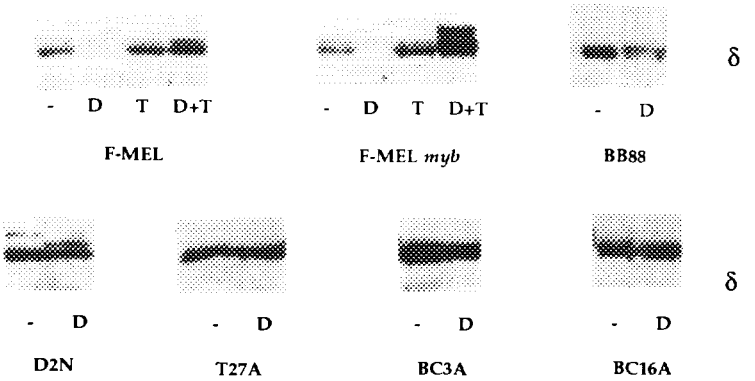

Fig. 3. Western analysis of PKC δ expression in Friend-virus-immortalized cell lines. Labelling is as in Fig. 1.

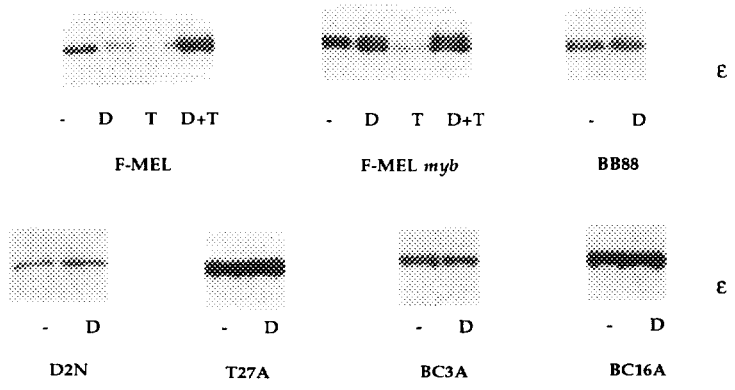

Fig. 4. Western analysis of PKC ε expression in Friend-virus-immortalized cell lines. Labelling is as in Fig. 1.

differentiation of F-MEL cells was associated with a drop in PKC δ. This was not seen in treatment of lines naturally resistant to differentiation. However, DMSO treatment of F-MEL *myb* cells still resulted in the same reduction of PKC δ as displayed by their differentiation-competent parental line, F-MEL. Thus, modulation of PKC δ may play a role in transmitting a signal to the nucleus during induced differentiation. This signal is unaffected by high levels of $p75^{myb}$.

Figure 4 shows that expression of PKC ε was downregulated approximately 50% on DMSO induced differentiation in the differentiation competent F-MEL cells. This was not seen in any other of the lines including the slightly differentiation competent BB88 or, in contrast to experiments with PKC δ, the F-MEL myb line. Thus, the constitutive expression of c-*myb* within F-MEL myb cells has achieved the same condition that exists within the naturally resistant lines with respect to PKC ε expression. This would indicate that if PKC ε downregulation is causally related to differentiation, that it lies downstream of c-*myb* and is perhaps regulated directly or indirectly by it.

PKC ζ is expressed at detectable levels and is modulated somewhat between samples, but in no apparent manner. Figure 5 shows comparable levels in F-MEL cells before and after DMSO treatment with a relatively elevated level on TPA treatment. F-MEL*myb* cells express relatively more PKC ζ than their parental counterparts.

Finally, the expression of one of the more recently discovered isoforms, PKC θ, could be key to the potential of a cell line to undergo erythroid differentiation. PKC θ is expressed only in F-MEL, BB88 and F-MEL*myb* cells (Fig. 6)—cells which are or have been competent for differentiation. None of the cell lines naturally resistant to differentiation showed detectable levels of this isoform. In additional experiments, we have determined that PKC θ is also

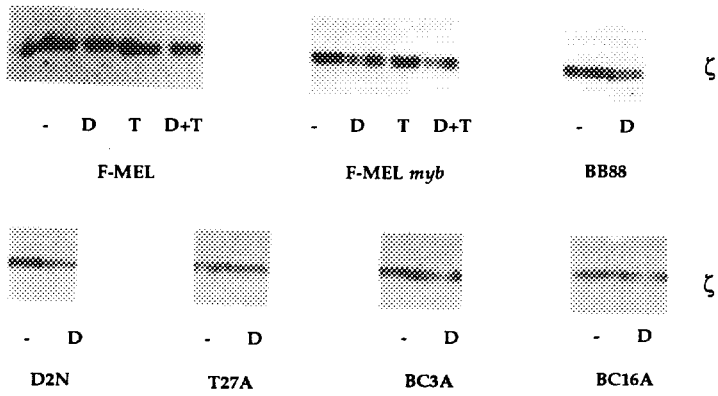

Fig. 5. Western analysis of PKC ζ expression in Friend-virus-immortalized cell lines. Labelling is as in Fig. 1.

expressed in K562 cells, another line capable of differentiation along the erythroid lineage. Other lines such as EL4, THP-1 and M1 do not express PKC θ. The isoform showed the highest level of expression in F-MEL and BB88 and was detected at a lower level in F-MEL*myb*. Downregulation of PKC θ occurred on DMSO treatment and was not affected by the presence of the constitutive c-*myb* in F-MEL*myb*. These results suggest its presence may be necessary for a cell to differentiate along the erythroid lineage, and if so, it probably lies at a point upstream of c-*myb* in the signal transduction event.

DISCUSSION

Substantial evidence exists indicating a role for both c-*myb* and PKC in the differentiation process of hematopoietic cells. Signal transduction pathways are thought to involve PKC by modulation of the activity of one or more of the isoforms. This modulation can occur by means of directly affecting the turnover rate of individual protein molecules, changes in the intracellular location or by changes in the absolute levels of protein isoforms (4). In efforts to help ascertain which isoforms are involved in this process, previous studies have utilized comparisons between cell lines with high and low levels of response to inducing agents (8-10). We have measured

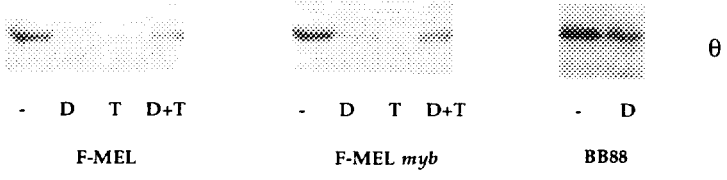

Fig. 6. Western analysis of PKC θ expression in Friend-virus-immortalized cell lines. Labelling is as in Fig. 1.

relative protein levels of $p75^{myb}$ and PKC isoforms in erythroleukemia cells before and after DMSO-induced differentiation. We have compared cell lines with high, low and zero levels of differentiation, as assessed by hemoglobin synthesis. We have concentrated on relative levels of isoforms and included one of the newly discovered isoforms, PKC θ, which is as likely a candidate as any of the other isoforms to play a role in differentiation. These comparisons have revealed several insights regarding these elements in the signal transduction pathway of differentiation.

Among these are that TPA, which blocks the DMSO-induced differentiation of F-MEL cells, does not block the downregulation of c-*myb*, which is necessary for differentiation. This suggests that TPA is modulating an element downstream of c-*myb* in the signal transduction pathway. These experiments also revealed that downregulation of $p75^{myb}$ is achieved in part by influencing the degradation of the protein or its mRNA as indicated by the fact that exogenously expressed $p75^{myb}$ was also downregulated. In addition to $p75^{myb}$, downregulation of three PKC isoforms, δ, ε and θ was also associated with DMSO-induced differentiation. Constitutive c-*myb* expression prevented downregulation of PKC ε, but not PKC δ and θ suggesting that levels c-*myb* may lie upstream of PKC ε and downstream of PKC δ and θ. $p75^{myb}$ also appears to influence the absolute level of PKC α with lower levels being present in the cells with the highest levels of $p75^{myb}$, the FMEL*myb* cell line.

These results help to characterize the involvement of PKC in hematopoietic differentiation. They also provide a framework to base future investigations. Further characterization of the α, δ, ε and θ isoforms is called for in regards to subcellular location during induction. In addition, the results suggest that exogenous expression of the isoforms δ, θ and perhaps α would most probably further elucidate the role of PKC in this process.

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