Down-Regulation of Human Protein Kinase C α Is Associated With Terminal Neutrophil Differentiation

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We have established an RNase protection method to quantify the expression of mRNA for the human protein kinase C (PK-C) isoforms α, β1, β2, and γ. This was used to investigate whether each isoform is differentially expressed during the differentiation of hematopoietic cells. Myeloid and lymphoid cells express PK-C α, β1, and β2 mRNAs in various proportions. PK-C γ mRNA was detected in human brain, but not in hematopoietic cells. PK-C α mRNA decreases as HL-60 cells mature to a neutrophil phenotype in response to retinoic acid, but its abundance does not change during monocytic differentiation in response to vitamin D3. PK-C α mRNA and protein were undetectable in peripheral blood neutrophils, but are present in monocytes. The mRNAs for PK-C β1 and β2 isoforms increase during HL-60 differentiation and are expressed in both neutrophils and monocytes. Therefore, the PK-C α isoform is specifically down-regulated during human neutrophil terminal differentiation. These data suggest that mature neutrophil functions do not require the PK-C α isoform. © 1992 by The American Society of Hematology.

PK-C has been implicated in hematopoietic differentiation as well as proliferation. PK-C is a receptor for the tumor-promoting phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), which directly activate PK-C both in vitro and in vivo, and TPA is known to induce differentiation in myeloid leukemic cells, including HL-60 promyelocytic cells. PK-C is also implicated in the function of mature hematopoietic cells, for example agonist-induced activation of the respiratory burst in neutrophils. In view of the possibly central role of PK-C in hematopoietic cell regulation, attempts have been made to characterize the PK-C profiles of hematopoietic cells at different stages of differentiation. It is difficult to obtain sufficiently pure normal hematopoietic precursors in adequate amounts at different stages of differentiation. Therefore, these studies have largely been performed on cell lines which have characteristics similar to a particular cell lineage arrested at a particular stage of differentiation. HL-60 cells are commonly used as a model of myeloid differentiation, since they can be induced to differentiate either to a neutrophil phenotype with retinoic acid or to a monocytic phenotype with dimethylsulfoxide (Me2SO). However, the differentiation is not complete, as the cells lack secondary granule components and the degree of apparent differentiation is dictated by the parameters chosen to measure a particular phenotypic characteristic.

Early studies on PK-C isoforms assayed for peaks of calcium and phospholipid-dependent kinase activity in fractions eluted from hydroxyapatite or diethylaminoethyl (DEAE)-cellulose chromatography columns. Antibodies have also been raised against the different isoforms and used in immunoprecipitation and immunoblot analysis. However, there are conflicting reports of the isoforms expressed, for example PK-C γ protein was reported to be present in HL-60 cells using immunochromatographic techniques, but was not detected by column chromatography. PK-C α activity and PK-C α protein were reported to be present in human neutrophils, but not in bovine neutrophils. In HL-60 cells induced to differentiate with retinoic acid, an increase in PK-C α protein was reported by one group, but a decrease in PK-C α activity on column chromatography by others. In addition, data are not available correlating differences in PK-C mRNA with levels of PK-C protein.

We have therefore established a highly sensitive and specific RNase protection assay to investigate the expres-
sion of PK-Cα, β1, β2, and γ mRNA in human myeloid and lymphoid cell lines, including HL-60 cells induced to differentiate with a variety of agents, and in normal human neutrophils, monocytes, lymphocytes, and platelets. This has been supplemented by Western blotting to correlate the findings on PK-Cα mRNA with PK-Cα protein.

MATERIALS AND METHODS

**TF-1, K562, HL-60, U937, Daudi, and Jurkatt Cells**

TF-1 is a multi-growth factor-dependent erythroleukemic cell line committed to the erythroid lineage. K562 is another erythroleukemic cell line, but exhibits characteristics of both immature myeloblasts and erythroblasts. HL-60 is a promyelocytic cell line and U937 a monoblastic cell line. Daudi is a B-lymphoid cell line and Jurkatt is a T-lymphoid cell line.

**Induction of HL-60 Differentiation**

Stock solutions of retinoic acid (Sigma Co Ltd, Poole, Dorset, UK; 33 nmol/L in 1:1 Me2SO/ethanol) and 1,25-dihydroxycholecalciferol (vitamin D3, kind gift of Roche Products Ltd, Welwyn Garden City, Hertfordshire, UK; 25.8 nmol/L in ethanol) were supplemented with 4 ng/mL of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Behringwerke, Marburg, Germany). K562, U937, Daudi, and Jurkatt cells were grown at 0.5 to 2.5 x 10^6 cells/mL in RPMI/10% fetal calf serum (FCS; Gibco-BRL, Uxbridge, Middlesex, UK) supplemented with 4 ng/mL of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Behringwerke, Marburg, Germany). K562, U937, Daudi, and Jurkatt cells were grown at 1 to 8 x 10^6 cells/mL in RPMI/5% FCS and HL-60 cells in RPMI/10% FCS. All cells were grown in a fully humidified atmosphere of 5% CO2.

**Preparation of Primary Hematopoietic Cells**

**Neutrophils.** A 45-ml sample of blood from a healthy volunteer was anticoagulated with 10 nmol/L of EDTA, pH 7.4, and centrifuged (150g, 20°C for 10 minutes to separate platelet-rich plasma (PRP), which was removed by aspiration and discarded. Platelet contamination of the subsequent cell preparation was therefore minimized. The volume was restored to 50 mL by adding RPMI containing 10 mmol/L EDTA and 2% FCS, and erythrocytes were sedimented with dextran 500 (0.55% vol/vol). The leukocyte-rich plasma (LRP) was layered on a single cushion of Lymphoprep (Nycomed Pharma AS, Oslo, Norway), and the neutrophils were separated from mononuclear cells by centrifugation at 800g, 20°C for 20 minutes. Remaining erythrocytes were removed by hypotonic lysis and the neutrophil pellet was resuspended in RPMI/EDTA/FCS. The total neutrophil yield was at least 5 x 10^7 from 45 mL of venous blood and had a purity of greater than 90% (Leishman staining). Contamination with monocytes was less than 4%.

**Monocytes.** Monocytes were prepared by centrifugation on a cushion of "Nycodenz monocytes" (Nycodenz) with the following modifications to the manufacturers instructions: 6-ml aliquots of LRP, prepared as described above, were layered onto 3 mL of Nycodenz monocytes in 14-mm diameter tubes (Falcon, Becton Dickinson and Co, Plymouth, UK), and then centrifuged at 600g, 20°C for 15 minutes. The upper clear plasma down to approximately 3 mm above the interphase was discarded. The interphase and just over half of the underlying Nycodenz monocytes was diluted to at least twice its volume with RPMI/EDTA/FCS and centrifuged at 600g for 10 minutes. The monocyte pellet was washed with RPMI/EDTA/FCS to remove contaminating platelets and resuspended in 5 mL of RPMI/EDTA/FCS. The total yield of monocytes varied greatly between individuals, although between 1 and 2.5 x 10^6 were usually obtained from 90 mL of venous blood. The purity was 90% with some contaminating lymphocytes.

**Lymphocytes.** A freshly removed human tonsil was obtained at tonsillectomy and the cells were extracted into RPMI/2%FCS by finely chopping and teasing out the cells with a scalpel blade. The resulting cell preparation was purified by centrifugation on Lymphoprep (600g, 20°C for 30 minutes) to obtain a population of mixed lymphocytes.

**Platelets.** PRP was prepared as described above, but only the top half of the PRP fraction (~3.5 mL) was taken for preparing RNA to minimize leukocyte contamination. The ratio of platelets to leukocytes in this preparation was greater than 26,000:1, which compares favorably with previously described methods. The total number of platelets obtained from 90 mL of blood was approximately 3.6 x 10^10. The platelets were pelleted by centrifugation of the PRP (2,000g, 20°C for 10 minutes). The pellet was resuspended directly into 3 mL of 4.23 mol/L guanidinium isothiocynate (GTC) for preparing RNA.

**Preparation of RNA**

Human fetal brain tissue was obtained with full consent and ethics committee approval at therapeutic termination of 8 to 14 weeks' gestation. Approximately 1 g of tissue was frozen by placing in dry ice/ethanol and then pulverised to a fine powder in a pre-cooled steel mortar and pestle. The homogenized tissue was transferred immediately into 10 to 20 mL of GTC and mixed thoroughly on a bench vortex. The resulting homogenate was aspirated through a 23-gauge needle four to six times to shear the DNA in the sample.

RNA was prepared from brain or cell pellets as described by Chirgwin et al. The yield was quantified by measuring the optical density at 260 nm (1 OD260/mL = 40 μg RNA in 1 mL). Typical RNA yields were 30 to 35 μg from 7 x 10^6 neutrophils, 30 μg from 1 to 2 x 10^7 monocytes and lymphocytes, 10 μg from platelets separated from 100 mL of peripheral blood, 500 to 700 μg from 1 g of human fetal brain, and 70 to 90 μg from 10^7 uninduced HL-60
cells. HL-60 cells differentiated with retinoic acid and vitamin D₃ yielded 40 to 50 μg/10⁷ cells.

**RNase Protection**

An RNase protection method was established essentially as described by Sambrook et al. and, unless otherwise stated, all genetic manipulations were as described within.

**Construction of probes.** All PK-C cDNAs were provided by Dr Peter Parker, Ludwig Institute, London. The cDNA encoding PK-C α was a full-length bovine cDNA, which was cloned in pSP65 (Promega Ltd, Southampton, UK). The bovine sequence was aligned with the known human cDNA sequence using VAX/VMS Version 4.6 P.L.R. software (S.E.R.C. Daresbury, Warrington, UK). The best homology was at the 5' end and a 138-bp fragment from a BstH II/NcoI digest (171 to 309) was chosen, since it was completely homologous to the human sequence apart from a single base mismatch at position 272. This fragment was blunt-ended and cloned into the Smal site in the polylinker in pGEM 2 (Promega). DNA sequence analysis confirmed the presence of the insert in the vector in an antisense orientation to the SP6 transcription promoter.

Both PK-C β1 and β2 full-length cDNAs were of human origin cloned in MT80. The two β isoforms have an identical sequence apart from the terminal 150 nucleotides and are thought to be derived by alternate splicing. The sequences chosen as probes for the β isoforms were selected from the nonidentical regions, and included part of the coding and noncoding regions. PKC β1 probe was a 238 bp TaqI/HindIII fragment from 2026 to 2264 cloned directionally in the sense orientation into AccI/HindIII cut pGEM 2. PK-C β2 probe was a 104 bp HindIII/EcoRI fragment from 1997 to 2101 cloned directionally in the sense orientation into HindIII/ EcoRI cut pGEM (Promega). DNA sequencing confirmed the presence of the insert in the expected orientation.

The starting cDNA for PK-C γ was a chimaeric construct containing a 300 bp fragment from BamHI to ApaI (165 to 465) of the human cDNA at the 5' end, which was fused with the bovine cDNA at the 3' end and cloned in pSP65. A 266-bp fragment was obtained by cutting the plasmid with TaqI, which contained human sequence from BamHI to TaqI (165 to 418) and a small segment (14 bp) of the polylinker from the original plasmid. This was cloned into the AccI site in the polylinker of pGEM2. DNA sequence analysis confirmed the presence of the insert in a sense orientation.

The DNA templates to be used in transcription reactions were linearized and then cleaned by phenol/chloroform extraction and ethanol precipitation. Finally, the DNA was resuspended in water at a concentration of 0.2 μg/μL. Between 0.5 and 1 μg of DNA was used for each transcription reaction.

**Transcription of the RNA probe.** The DNA template was aliquoted into a 500-μL microfuge tube. The following were then added: 2 μL of 5x transcription buffer (200 mmol/L Tris-HCl, 30 mmol/L MgCl₂, 10 mmol/L spermidine, pH 7.5), 1 μL of 10x rNTF% (5 mmol/L with respect to UTP, ATP, and CTP [Pharmacia, Uppsala, Sweden]), 0.4 μL of 250 mmol/L dithiothreitol, 0.5 μL of RNasin (Promega), 50 μCi of [³²P]GTP (>400 Ci/mmol/L, Amersham PB10161; Amersham International, Amersham, Buckinghamshire, UK), and 1 μL of the appropriate RNA polymerase (SP6 RNA polymerase for PK-C α, T7 RNA polymerase for PK-C β1, β2, and γ, Promega; 10 to 20 U/μL). The transcription reaction was performed in a total reaction volume of 20 μL at 37°C for 90 minutes. The DNA template was then digested with RNase-free DNase and the radioactive probe was then extracted with phenol/chloroform, precipitated, and resuspended in 100 μL of TES (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.1% sodium dodecyl sulfate [SDS], adjusted to pH 7.5). The specific activity of the probes was approximately 10⁵ cpm/μL probe. We have determined that the probes could be used without further purification through a polyacrylamide gel (data not shown).

**Hybridization in vitro.** Between 2 and 20 μg of total RNA extracted from cells or tissue was freeze-dried and resuspended in 25 μL of freshly made hybridization buffer (80% deionized formamide, 40 mmol/L NaPiPES, pH 6.4, 1 mmol/L EDTA, 400 mmol/L NaCl, 0.1% SDS). Twenty micrograms of RNA was used as a negative control. One microliter of the specific PK-C probe was added to each hybridization reaction. Actin probe was also added to the hybridization to control for the possible variation in aliquoting (“loading”) of RNA in the reaction. The actin fragment, cloned in an SP6 transcription vector, was provided by Dr Linda Penn, ICRF, London. However, the mRNA for actin was found to be expressed at much higher levels than for PK-C, and therefore the radioactive actin probe was further diluted 1/50 in TES containing 2 ng of unlabeled actin probe with 5 μg tRNA as carrier. This ensured that the actin probe was always in excess in the reaction. One microliter of the final diluted actin probe was used for each hybridization.

The hybridization reaction was first heated at 85°C for 10 minutes to denature the RNA and then transferred immediately to a waterbath at 30°C for 16 hours to allow hybridization to occur.

**RNase reaction.** After hybridization, the reaction was incubated at 30°C for 60 minutes with 350 μL of freshly made RNase solution (10 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 300 mmol/L NaCl, 40 μg/mL RNase A, and 2 μL/mL RNase T1 [Boehringer Mannheim, Lewes, East Sussex, UK]) to digest any unprotected single-stranded RNA species. The reaction was terminated by adding 10 μL of 20% SDS and 10 μL of freshly made proteinase K (10 mg/mL in TE [10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA], pH 7.5) and then incubating at 37°C for 15 to 30 minutes.

**Extraction of protected probe(s).** All traces of RNase activity were removed from the reaction by extracting with an equal volume (400 μL) of phenol chloroform. The protected probe was coprecipitated in ethanol with 1 μL of glycerol (20 mg/mL, Boehringer Mannheim). The RNA pellet was dried and resuspended in 15 μL of RNA loading dye (80% formamide/1x TBE [89 mmol/L Tris-borate, 89 mmol/L boric acid, 2 mmol/L EDTA], pH 8.3, 0.1% wt/vol xylene cyanol, 0.1% wt/vol bromophenol blue).

**Separation of protected probe by denaturing polyacrylamide gel electrophoresis.** The probe was boiled for 5 minutes and immediately quenched in ice water before loading on a 4% polyacrylamide/7.5 mol/L Urea/TBE gel (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK; 16 cm × 20 cm × 0.75 cm, Protein II xi System). Electrophoresis was at a constant current of 42 mA.

**Autoradiography.** The dried gel was autoradiographed on preflushed Kodak X-OMAT AR or RP film (Sigma) with a single intensifying screen (Fuji, Genetic Research Instrumentation Ltd, Dunmow, Essex, UK). A detectable signal was obtained for PK-C α, β1, and β2 probes after 5 to 7 hours' exposure on X-OMAT AR film and up to 12 hours for PK-C γ. The exposure times necessary using the slower film X-OMAT RP were 16 to 20 hours for PK-C α, β1, and β2, and 48 to 72 hours for PK-C γ.

**Scanning densitometry.** Autoradiograms were scanned with a Hoefer GS-300 scanning densitometer (Hoefer Scientific Instruments, Newcastle, Staffordshire, UK) and the data were analyzed with Hoefer GS-370 densitometry software on an Apple Macintosh computer (Apple Computer Ltd, Uxbridge, Middlesex, UK). The area under the peak due to the PK-C isoform was determined and this was then divided by the area under the actin peak in the same sample. This ratio of PK-C isoform to actin mRNA was further corrected to account for the total amount of mRNA isolated per cell.
which decreases up to 50% during HL-60 differentiation (data not shown). The relative abundance of the PK-C isoform mRNA in each sample is thus corrected for the loading (relative to actin mRNA) and is expressed on a per cell basis. To compare the relative abundance of the four PK-C isoform mRNAs in any particular sample, a further correction has to be made for the specificity of each protected probe used. This depends on the number of guanosine residues in the protected probes, since $^3$P-GTP is used in the transcription reaction (PK-C α, 35; PK-C β1, 54; PK-C β2, 31; PK-C γ, 74), assuming incorporation of radioactivity into each probe is linear.

Controls for RNase protection. Twenty micrograms of total cellular RNA from human fetal brain was used as a positive control for the RNase protections, since all four isotypes are known to be expressed in this tissue.² Twenty micrograms of tRNA, and an aliquot of the probe processed in the absence of RNA, were used as negative controls. A PK-C β1 probe transcribed using the constructed pGEM vector, of exactly the same sequence as the mRNA of interest, failed to hybridize with RNA from human brain (data not shown), confirming the absence of nonspecific protection and “cross-talk” of SP6 and T7 transcription promoters.

Western Blotting

The methods used are as described previously,⁴ except that the total cell extract was separated by electrophoresis through 7% polyacrylamide SDS gels, and all antibody and wash solutions also contained 0.1% Triton X-100. The anti-PK-C α antibody used was an antipeptide antibody raised in rabbit. This was a generous gift from Dr. Peter Parker, Ludwig Institute, London. For peptide competition, the peptide was added to the antibody at room temperature for 30 minutes before use. 125I-protein A (Amersham, 1 µCi/mL final) was used as the second layer.

Western blotting using anti-PK-C β1 and β2 antibodies resulted in excessive nonspecific signals, and therefore data on PK-C β1 and β2 protein are not available.

RESULTS

Sensitivity of PK-C RNase Protection

To quantify the sensitivity of our protection assay, we transcribed an RNA fragment identical to the mRNA sequence for PK-C β1. The amount of this RNA was quantified by measuring the optical density at 260 nm. Serial dilutions of the RNA from 2 ng to 1 fg were then made in TES containing 5 µg/µL of tRNA as carrier. The RNA was then hybridized with a standard amount of the PK-C β1 probe as described above. The least amount of specific mRNA that could be detected by the PK-C β1 probe was 1 pg after 20 hours exposure to Kodak X-OMAT RP film, and less than 5 hours on Kodak X-OMAT AR film (Fig 1). This is equivalent to approximately 10⁷ copies of specific mRNA in the reaction. The sensitivity of the RNase protection compares well with quantitative PCR protocols.⁴⁹,⁵⁰ Similar results were obtained with PK-C α, β2, and γ (data not shown).

Reproducibility of RNase Protection

To assess the reliability of our assay, the PK-C β1 probe and the actin probe were hybridized with a standard amount (20 µg) of U937 total cellular RNA in 10 separate reactions. The coefficient of variation of PK-C β1/actin ratio was 17% (mean ± SD, 1.16 ± 0.20, n = 10). In a similar experiment using total cellular RNA from HL-60 cells, in which the level of expression of PK-C β1 is approximately half that of U937 cells, the coefficient of variation was 27% (mean ± SD, 0.62 ± 0.17, n = 10).

PK-C mRNA Expression in Human Hematopoietic Cell Lines

The abundance of PK-C α, β1, β2, and γ mRNA in the myeloid cell lines TF-1, K562, HL-60, and U937, and the lymphoid cell lines Daudi and Jurkatt, was determined by RNase protection. PK-C γ mRNA was not detected in any cell line, but was present in human fetal brain. PK-C β1 and β2 mRNA were present in all cell lines in generally similar ratios. PK-C α mRNA was present in the lymphoid cell lines and all myeloid cell lines except U937 (see ref 53).

PK-C mRNA Expression in HL-60 Cells Induced to Differentiate With Retinoic Acid, MeSO, and Vitamin D₃

As retinoic acid induces differentiation to a granulocytic phenotype,⁶ and vitamin D₃ induces a monocytic phenotype,⁸ HL-60 cells were exposed to these agents and changes in the mRNA for PK-C α, β1, β2, and γ were determined (Table I). There was a significant reduction in PK-C α mRNA when HL-60 cells differentiated in response to retinoic acid, but no change in PK-C α mRNA occurred during treatment with MeSO or vitamin D₃.

Table I. PK-C mRNA Isoform Changes in HL-60 Cells in Response to Induction of Differentiation With Retinoic Acid and Vitamin D₃

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Relative Copy No. of PK-C Isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
</tr>
<tr>
<td>Control</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>0.9 ± 0.5*</td>
</tr>
<tr>
<td>(62% ± 10%)</td>
<td>(339% ± 204%)</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>(93% ± 17%)</td>
<td>(189% ± 76%)</td>
</tr>
</tbody>
</table>

HL-60 cells were induced to differentiate with retinoic acid and vitamin D₃ for 5 to 7 days, and probed for PK-C α, β1, β2, and γ by RNase protection. Twenty micrograms of total cellular RNA was used for protection with each PK-C probe, together with actin probe as internal control. Relative copy numbers per cell of PK-C α, β1, and β2 mRNA were determined in five separate experiments by scanning densitometry (see Experimental Procedures). The mean ± SD values are given. The significance of the differences observed were analyzed by paired t test. In addition, the results of each experiment were expressed as a percentage of control HL-60 (undifferentiated, actively dividing cells) and the mean ± SD values are also given below.*P < .05.
when differentiated with vitamin D3. Retinoic acid caused a twofold to threefold increase in PK-C β1 and β2 mRNA. Vitamin D3 induced a similar increase in the β isoforms, but the data are statistically significant for PK-C β1 only. HL-60 cells induced to differentiate to a neutrophil phenotype with Me2SO for 7 days showed changes in the PK-C mRNAs similar to those caused by retinoic acid, but were not statistically significant (data not shown).

To investigate the kinetics of the changes in the PK-C isoforms, HL-60 cells were induced to differentiate with retinoic acid over a period of 3 days. The signal due to PK-C α (Fig 2A) shows little change with retinoic acid treatment over 3 days. In addition, the signal due to actin is also relatively constant. Hence, the ratio of the signals, PK-C α to actin, remains constant with differentiation when a standard amount of RNA is used in the reaction. However, the amount of RNA extracted decreases during differentiation with retinoic acid and, therefore, after correction of this ratio to account for the diminution in total RNA (see Experimental Procedures), there is a reduction of nearly 40% in PK-C α mRNA per cell in this experiment. The levels of PK-C β1 mRNA (Fig 2B) and β2 mRNA (Fig 2C) per cell show a twofold to threefold increase with retinoic acid treatment. The decrease in the level of PK-C α mRNA and the increase in PK-C β1 and β2 mRNA is complete within 2 days and there is little further change thereafter. PK-C γ mRNA was not detected in HL-60 cells treated with any inducer (data not shown).

PK-C mRNA Expression in Primary Human Hematopoietic Cells

To compare the results obtained with partially differentiated HL-60 cells with those obtained from terminally differentiated cells, the PK-C isoforms expressed in neutrophils and monocytes from peripheral blood were determined. Only a qualitative assessment could be made of the isoforms present in these terminally differentiated cells, since little or no signal was obtained for actin used as an internal control (Fig 3). PK-C β1 (Fig 3B) and β2 (Fig 3C) mRNA are both expressed in these primary cells, but PK-C γ (Fig 3D) mRNA was undetectable. PK-C α mRNA was

\[ \text{PK-C mRNAs} \]

\[ \text{Actin} \]

\[ \text{PK-C α, β1, or γ} \]

\[ \text{Actin} \]

\[ \text{PK-C β1 or β2} \]

\[ \text{Actin} \]

\[ \text{PK-C β2} \]
PK-C α IN NEUTROPHIL DIFFERENTIATION

Fig 3. PK-C isoform mRNA in human neutrophils and monocytes. Ten micrograms of total cellular RNA was used to probe for PK-C α, and 5 μg for PK-C β1, β2, and γ mRNA in: m, monocyte; n, neutrophil. Actin probe was used as internal control. (A) PK-C α, (B) PK-C β1, (C) PK-C β2, (D) PK-C γ. Control samples were: b, human fetal brain; t, tRNA; c, no RNA.

not detected in neutrophils, but was present in monocytes (Fig 3A). The differential expression of PK-C α mRNA in neutrophils and monocytes was confirmed in cells from three normal individuals. Since the magnitude of the signal due to PK-C α mRNA was similar in equivalent amounts of RNA from monocytes and lymphocytes, the presence of 10% lymphocyte contamination of the monocyte preparation was not a significant contributor to the signal obtained in monocytes (data not shown). In addition, platelets also contain PK-C α, β1, and β2 mRNA (data not shown), but significant platelet contamination of the neutrophil and monocyte preparation was prevented (as outlined in Exper-
PK-C α Protein Expression in Primary Human Hematopoietic Cells and HL-60 Cells

Expression of PK-C α protein in HL-60 cells induced to differentiate to a granulocytic phenotype with retinoic acid was investigated by Western blot analysis to determine if obtained in the primary cells. Differentiate to a granulocytic phenotype with retinoic acid.

Cells and HL-60 Cells

Figure 4 shows a paradoxical increase in the amount of PK-C α protein per cell after retinoic acid treatment even though the amount of PK-C α mRNA per cell decreased during this period (Table 1, Fig 2A). The mechanism by which this occurs has not been characterized. However, neutrophils have no detectable PK-C α protein, whereas a strong signal was detected in monocytes (Fig 5). These data suggest that down-regulation of PK-C α occurs as a late event in neutrophil differentiation.

Retinoic acid treatment of HL-60 cells caused an induction of a protein of 47,000 M, (Fig 4), which also occurred as a consequence of vitamin D3 or Me2SO induction (data not shown). This protein is probably the p47 component of the respiratory burst pathway, which is induced by all these agents, and which shares a common epitope with PK-C α (C.G. Teahan and A.W. Segal, University College, London, personal communication). It is unlikely to be a degradation product of PK-C α, since a band of identical molecular weight is detected by a monoclonal antibody (AW SS) specific to the 47,000 M, respiratory burst protein, which does not bind to PK-C α (data not shown).

DISCUSSION

Investigation of the relative expression of PK-C mRNAs has been hampered by the fact that their abundance in the cell is extremely low. We have therefore established a highly sensitive RNase protection assay specific for human PK-C α, β1, β2, and γ mRNA. The method enables reliable quantitation and as little as one copy per cell of specific mRNA can be detected in RNA from 10⁷ cells (Fig 1). We have expressed our results, where possible, as relative copy number per cell of the specific mRNA of interest, taking into account the significant reduction of the total RNA extracted from cells at different stages of differentiation. The results therefore reflect the physiologically relevant copy number of the different isoforms per cell, as opposed to their concentration in a given amount of total cellular RNA.

To determine whether differential expression of PK-C isoforms occurs at different stages of hematopoietic differentiation, we initially screened a number of myeloid and lymphoid cell lines. Cell lines of both myeloid and lymphoid lineages express mRNA for PK-C α, β1, and β2, but not γ. HL-60 cells were then used as a model to study whether changes in expression occur with differentiation to a neutrophil or a monocyte phenotype. There was an increase in PK-C β1 and β2 mRNA when these cells were induced to differentiate with retinoic acid or vitamin D3 (Table 1). In contrast, there was a reduction in PK-C α mRNA when

![Fig 4. Change in PK-C α protein in HL-60 cells induced to differentiate with retinoic acid. Western blot analysis, with antibody to PK-C α, of total cell protein samples from approximately 2 x 10⁶ HL-60 cells at 1, 2, and 3 days after treatment with retinoic acid. (Right) A control experiment in which PK-C α antibody was preincubated with a specific PK-C α peptide (+), or was used without prior preincubation with the peptide (−). The blot is the same as that on the left, but the HL-60 sample used (day 5 retinoic acid) was from a separate experiment. PK-C α protein is not detected when the PK-C antibody is preincubated with a specific PK-C α peptide (+), confirming the specific identity of the signal. Other samples: s, PK-C α standard (partially purified mixture of PK-C α, β, and γ, Peter Parker, Ludwig Institute, London); m, monocyte.

![Fig 5. PK-C α protein in human neutrophils and monocyes. Human neutrophils and monocyes were used for Western blot analysis using anti-PK-C α antibody; n, neutrophil; m, monocyte; b, human fetal brain. Preincubation of the PK-C α antibody with the PK-C α peptide competes out the PK-C α signal (shown as + competition).]
HL-60 cells were induced to differentiate to a neutrophil phenotype with retinoic acid, but not when induced to a monocyte phenotype with vitamin D₃. Previous studies have also shown an increase in PK-C α mRNA (with no separation into β1 and β2) in response to vitamin D₃. In addition, other studies show an increase in PK-C β protein and activity with retinoic acid. In contrast, an increase in PK-C α mRNA was reported to occur after retinoic acid treatment, but a reduction in PK-C α activity was reported by others. These apparent discrepancies underline the difficulty in studying families of molecules which share considerable homology, and emphasize the requirement for stringent specificity using any particular methodology. Our results with HL-60 cells, using the highly specific RNase protection method, suggested that down-modulation of PK-C α mRNA may be associated with neutrophil, but not monocyte, differentiation. Therefore, the PK-C isoforms expressed in terminally differentiated neutrophils and monocytes were determined. Neutrophils have previously been reported to express both PK-C β and α (in a ratio of 65% and 35%, respectively, of total PK-C), which were detected by column chromatography and immuno precipitation methods. In contrast, we have failed to detect PK-C α mRNA in neutrophils (Fig 3) from three normal individuals and no PK-C α protein was detected in neutrophils by Western blotting (Fig 5). However, monocytes do express PK-C α mRNA (Fig 3) and protein (Fig 5). The data on primary cells and on HL-60 differentiation would suggest that down-regulation of PK-C α is associated specifically with neutrophil, but not monocyte, differentiation.

Our data also suggest that PK-C α protein in HL-60 cells increases slightly during the early stages of differentiation with retinoic acid (Fig 4), as has been reported previously, despite a decreasing level of PK-C α mRNA. Therefore, this change in the levels of PK-C α protein must occur by posttranscriptional regulation. Absence of PK-C α protein in neutrophils suggests that it is down-regulated at a late stage in terminal differentiation, a stage not reached by the "retinoic acid–HL-60" model. Indeed, HL-60 cells do not show the characteristics of mature neutrophils and therefore only undergo partial differentiation along this lineage.

Neutrophils and monocytes are derived from a common progenitor cell and so the differential expression of PK-C α in these cells raises questions about its role in terminal differentiation and its function in mature monocytes. It is not known whether loss of PK-C α is an obligate step in neutrophil differentiation or is merely caused as a consequence of the differentiation process. However, it is clear that PK-C α cannot be required for mature neutrophil functions, such as respiratory burst activity. In support of this, we have shown previously that U937 cells, which also have no detectable PK-C α mRNA or protein, were able to develop respiratory burst activity after differentiation with gamma interferon.

In conclusion, we have demonstrated differential expression of PK-C α mRNA and protein in human neutrophils and monocytes, and this occurs during late terminal differentiation of the myeloid progenitor cell.

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