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Research Article

# Tyrosine phosphorylation of cytoplasmic proteins in proliferating, differentiating, apoptotic HL-60 cells and blood neutrophils

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**Abstract.** Two-dimensional electrophoretic analysis was used to assess quantitative and qualitative changes in the expression and tyrosine phosphorylation of cytoplasmic proteins of proliferating, differentiating HL-60 cells and mature human blood neutrophils. The total tyrosine phosphorylation level of cytoplasmic proteins appeared approximately constant during the pre-commitment period, i.e., 6–24 h after induction of differentiation by 700 nM all-*trans* retinoic acid. At the time of granulocytic phenotype formation (48–120 h), the total level of tyrosine phosphorylation of cytoplasmic proteins increased significantly. Tyrosine phosphorylation of cytoplasmic proteins in matured blood neutrophils was significantly lower than that of cytoplasmic proteins of HL-60 cells differentiated for 96 h with retinoic acid. Immunoblotting with anti-Erk2 and anti-phosphotyrosine monoclonal IgG2b$_k$ antibodies showed that Erk2 was expressed and tyrosine-phosphorylated at different levels in HL-60 proliferating cells and in cells at all stages of differentiation. Our data showed that tyrosine phosphorylation of cytoplasmic proteins in differentiating HL-60 cells changes dramatically during the period of phenotype formation and is accompanied by increasing activity of Erk2. An increasing number of apoptotic cells appeared in the differentiating HL-60 cell population during the granulocyte maturation stage (48–120 h of differentiation). The appearance at this time of differentiation of a new set of tyrosine-phosphorylated cytoplasmic proteins (also distinctive for apoptotic HL-60 cells mediated by etoposide) together with an increasing number of apoptotic cells in the differentiating population strongly suggests that these proteins are associated with the apoptotic process.

**Key words.** HL-60; maturation in vitro; neutrophils; apoptosis; Erk2.

The HL-60 cell line was originally isolated from a patient with promyelocytic leukemia [1]. These cells are arrested at the promyelocytic stage of development, but can be induced to differentiate to neutrophil-like cells by continuous incubation for instance with all-*trans* retinoic acid (RA) [2]. RA is a physiological metabolite of vitamin A, and acts through a subfamily of nuclear hormone receptors, RARs and RXRs (retinoid X receptors), which regulate the expression of target genes [3–5]. RA, along with its differentiation activity, causes apoptosis in the HL-60 cells [6–9]. Thus the cells not only differentiate but also subsequently die by pro-

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grammed cell death (apoptosis) between day 6 and 8 after induction of differentiation [10, 11]. Therefore, we used HL-60 cells for molecular analysis of the events occurring after treatment with RA, which should provide new information on the relationship between differentiation and apoptosis of granulocytes.

A differentiation program is initiated by transcriptional activation of defined genes and is closely linked to the molecular mechanisms that lead to withdrawal from the cell cycle. Whether a cell progresses through the cell cycle, enters the differentiated state, or becomes apoptotic depends on sets of proteins being activated or repressed in the cell. Although phosphotyrosines represent only a small proportion of the total phosphoamino acid content of eukaryotic cells, specific tyrosine phosphorylations or dephosphorylations have been shown to play a key role in the regulation of cell differentiation [12–18].

Many protein tyrosine kinases (PTKs) are involved in growth control and are implicated in cell transformation [19–23], where protein tyrosine phosphatases (PTPs) have also been shown to have important regulatory functions [24, 25]. Thus, the balance between the actions of PTKs and PTPs determine the levels of phosphorylation, which have appeared crucial for induction and maintenance of the differentiated phenotype of cells [26, 27]. RA-mediated granulocytic differentiation of hematopoetic cells is also accompanied by activation of Src and Syk family PTKs, intense expression of *fgr*, and weak expression of *lyn* and *fyn* genes [10, 28, 29]. Crkl has been identified as the major tyrosine-phosphorylated protein in chronic myelogenous leukemia neutrophils [30, 31]. The product of proto-oncogene *vav*, $p95^{vav}$, which is solely expressed in hematopoetic cells, is tyrosine-phosphorylated and is assumed to play an important role in signal transduction [32]. There have also been several reports of PTKs which are localized both in cytoplasm and the nucleus and become tyrosine-phosphorylated during cell transformation or differentiation. These include p140/c-Abl in HeLa cells [33], FER in various cell lines [34], and c-Fes in myeloid leukemia cells [35, 36]. Furthermore, granulocyte/macrophage-colony stimulating factor stimulation of undifferentiated and differentiated HL-60 cells results in phosphorylation and activation of STAT5 and MAP kinase [37]. The role of PTKs in apoptosis is not clear, since they can facilitate [38–42, 49] or inhibit [38, 43–49] apoptosis in different cell lines.

To elucidate the possible involvement of tyrosine-phosphorylated cytoplasmic proteins in signal transduction during granulocytic differentiation, we characterized quantitative and qualitative modifications of cytosolic proteins in proliferating and differentiated HL-60 cells and in mature neutrophils.

The results reported show that the total tyrosine phosphorylation level of cytoplasmic proteins is fairly constant during the first cell cycle after induction of differentiation (6–24 h). At the time of commitment and granulocytic phenotype formation (48–120 h), this level increases significantly, while it is drastically decreased in mature blood neutrophils. The phosphotyrosine content of PY-Erk2 is detectable 0.5 h after induction of differentiation and it significantly augments during the commitment and granulocytic phenotype formation periods (48–120 h). Immunoblotting with anti-Erk2 antibody shows that this protein is expressed in HL-60 cells, both during proliferation and at all stages of differentiation.

We suggest that the dramatic changes in protein tyrosine phosphorylations seen in differentiating HL-60 cells are associated with the formation of the differentiated granulocyte phenotype. At late stages of differentiation (72–120 h), some modified proteins appear to be related to the apoptotic process, since the number of apoptotic cells after the commitment stage of HL-60 cell differentiation gradually increases.

## Materials and methods

**Reagents.** RA, etoposide, nitroblue tetrazolium (NBT), phorbol 12-myristate 13-acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin, aprotinin, NaF, sodium orthovanadate ($Na_3VO_4$), DNase I, RNase A, Tris, EDTA, SDS, Nonidet P-40, Tween-20, 3 - [(3 - cholamdopropyldimethylammonio] - 1 - propanesulfonate (CHAPS), dithiothreitol (DTT), 2-mercaptoethanol, glycine, sucrose, urea, and glycerol were obtained from Sigma. Acrylamide, methylene-bisacrylamide (BIS), N,N,N,N′-tetramethylenediamine (TEMED), and protein molecular mass markers were obtained from Bio-Rad. Cell culture medium RPMI 1640 and fetal bovine serum (BSA) were obtained from Gibco BRL Life Technologies; penicillin and streptomycin were obtained from NordCell, Sweden; pharmalytes pH range 3–10 and a silver staining kit were obtained from Pharmacia Biotech. A colloidal coomassie stain kit was obtained from Novel Experimental Technology Novex. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Ig) was purchased from DAKOPAT. Anti-phosphotyrosine monoclonal $IgG2b_k$ antibodies and monoclonal anti-MAP kinase (Erk2) were obtained from UBI. An activated form of Erk2 (PY-Erk2) was obtained from Promega, the enhanced chemiluminescence Western blot detection system (ECL) was obtained from Amersham. Polymorphprep was purchased from Nycomed Pharma AS. Other chemicals used in this work were of analytical grade.

**Cell culture.** The human promyelocytic leukemia cell line HL-60 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% $CO_2$ humidified incubator at 37 °C. Cells were seeded at a level of $4 \times 10^5$ cells/ml and allowed to attain a maximum density of $1.5 \times 10^6$ cells/ml before being transferred to fresh medium. Cells were not used beyond passage number 60. Granulocytic differentiation was induced by treating $5 \times 10^5$ cells/ml with 700 nM RA. Stock solutions of 10 mM RA in 96% ethanol were stored at −20°C. The extent of differentiation was assayed by the ability of the cells to reduce NBT to insoluble blue-black formazan on stimulation with PMA [50]. One hundred microliters of cell suspension from the cultures was mixed with an equal volume of 0.2% NBT dissolved in phosphate-buffered saline containing 40 ng/ml PMA and further incubated at 37 °C for 30 min. NBT-positive cells were counted using a hemocytometer. At least 200 cells were scored for each determination and the number of NBT-positive cells was expressed as a percentage of the total number of viable cells as determined by the exclusion of 0.2% trypan blue. Differentiating populations containing no less than 50–60% differentiated cells were used. Cell viability was assayed by exclusion of 0.2% trypan blue, and the proportion of apoptotic cells was quantified using acridine orange and ethidium bromide staining according to Mercille and Massie [51].

**Induction of apoptosis.** Apoptosis was induced by treating logarithmically growing HL-60 cells with 68 μM etoposide (stock solution was 68 mM etoposide in DMSO). After 6 h, 80–85% of cells were scored as apoptotic using acridine orange and ethidium bromide [51].

**Isolation of cytosolic proteins.** Nuclei were isolated as described by Antalis and Godbolt [52] with some modifications. Cells were collected by centrifugation and washed twice in solution containing 0.1 M $NaH_2PO_4$-0.1 M $Na_2HPO_4$, 0.1 M NaCl, pH 7.5 (PBS), 10 mM NaF, and 1 mM $Na_3VO_4$. Then, $3 \times 10^7$ cells were resuspended in 5 vol of solution A (10 mM NaCl, 10 mM Tris/HCl, pH 7.5, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, and pepstatin, 10 mM sodium fluoride, 1 mM sodium orthovanadate) and kept for 15 min at 0 °C to swell. The cell suspension was shaken vigorously by hand and immediately mixed 1:1 (v/v) with solution A containing 0.6 M sucrose (solution B). The cell homogenates were then centrifuged at 1500*g* for 5 min. The supernatant corresponding to the cytosol fraction was clarified by centrifugation at 20,000*g* for 30 min and frozen at −76 °C.

**Isolation of granulocytes.** To obtain normal neutrophils, heparinized blood (25 ml) from healthy adult volunteers was layered on 20 ml Polymorphprep and 50 ml Lymphoprep gradient followed by centrifugation at 600*g* for 45 min at room temperature. The uppermost layer down to the granulocyte band was aspirated. The band with granulocytes was transferred to a tube, gently mixed with an equal volume of PBS, pH 7.3, and centrifuged at 600*g* for 10 min at room temperature. The cells were resuspended in 9 ml cold distilled water, 3 ml 3.4% PBS and 5 ml Krebs-Ringer phosphate buffer (KRG, pH 7.2) without $Ca^{2+}$ and sedimented at 200*g* for 10 min at 4–8 °C. The neutrophils were kept in a melting ice bath until used for cytosolic protein isolation.

**Preparation of protein samples for electrophoresis and isoelectrofocusing.** Protein samples for SDS/PAGE were prepared as follows: to 100 μl of the cytosolic protein fractions, 12.5 μl 0.5 M Tris/HCl, pH 6.8, 30 μl 10% SDS, 20 μl 0.5 M DTT, 20 μl glycerol, and 5 μl 0.5% bromphenol blue were added. The mixtures were boiled for 5 min and kept at −80 °C. Protein samples for isoelectrofocusing were prepared by mixing cytosol (in at least a 1:4 ratio) with sample solution containing 9 M urea, 2% w/v CHAPS, 1% w/v DTT, 0.8% w/v 2-D pharmalytes, pH range 3–10, and 0.01% w/v bromophenol blue.

**Gel electrophoresis.** The cytosolic proteins were resolved by SDS electrophoresis and two-dimensional gel electrophoresis (IEF/SDS). SDS electrophoresis was performed using a 7–15% polyacrylamide gradient gel with Tris-glycine electrophoresis buffer.

In some experiments, two parallel gels were used if needed. An Immobiline DryStrip Kit, pH range 3–10, and Exel Gel SDS, gradient 8–18% were used for two-dimensional electrophoresis, carried out according to the manufacturer's instructions (Pharmacia Biotech). For analysis of total cytosolic proteins, SDS/PAGE and 2-DE gels were stained using either the Pharmacia Silver Staining Kit or the NOVEX Colloidal Coomassie Stain Kit (Novel Experimental Technology Novex) according to the manufacturer's instructions.

**Immunoblotting analysis.** After SDS or two-dimensional electrophoresis, proteins were transferred to an Immobilon PVDF transfer membrane (Millipore) and then blocked with 5% BSA dissolved in PBS containing 0.1% Tween-20 by incubation overnight at 4 °C. After washing in PBS-Tween-20, the filters were probed with anti-phosphotyrosine antibody, as described below. For tyrosine-phosphorylated protein analysis, the membranes were incubated for 1 h at room temperature with a 1:4000 dilution of anti-phosphotyrosine antibodies ($IgG2b_k$) in PBS-Tween-20, and washed four times for 30 min with PBS-Tween-20. After washing, the membranes were incubated further with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in PBS-Tween-20 for 1 h at room temperature. This

washing procedure was repeated, and immunoreactive bands were detected by enhanced chemiluminescence following the manufacturer's instructions. For detection of Erk, the same membranes were used after stripping and reprobing according to the ECL Western blotting protocol (Amersham, Life Science). Briefly, to remove the primary and secondary antibodies from the membranes, they were submerged in stripping buffer consisting of 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris/HCl, pH 6.7, and incubated at 50 °C for 30 min with occasional agitation. The membranes were then washed for 2 × 15 min at room temperature using large volumes of wash buffer (PBS-Tween-20) and reprobed.

## Results

**HL-60 cell differentiation, viability and apoptosis after treatment with RA.** Typically, the number of differentiated cells increased from 6–8% at 48 h to 50–55% at 120 h after induction of differentiation (fig. 1A). The viability of the cells remained above 95–90% up to 72 h and then decreased to 65% between 72 and 120 h (fig. 1C). The proportion of apoptotic cells in the differentiating HL-60 cell culture started to increase from the commitment stage (48 h), and at 120 h of differentiation formed 50–55% of the population (fig. 1B).

**Electrophoretic patterns of total and tyrosine-phosphorylated cytosolic proteins during HL-60 cell differentiation.** Cytoplasmic proteins were isolated from differentiating cells between 6 and 120 h after induction of differentiation and from control proliferating cells (fig. 2A). The electrophoretic patterns suggest that there were no significant qualitative differences in cytoplasmic proteins of control and differentiating cells.

We found, however, that the total level of protein tyrosine phosphorylation became more intensive in the first 48 h after differentiation, and then further increased markedly during the commitment period (48–72 h) and in the granulocyte phenotype formation period (96–120 h) (fig. 2B). For constitutively tyrosine-phosphorylated proteins, phosphorylation was proportional to the number of differentiating cells in the population and was maximal around 96–120 h after induction.

**Expression and activity of Erk2 after induction of HL-60 differentiation.** The total level of Erk2 and active Erk2 were estimated in four immunoblotting experiments using monoclonal anti-MAP kinase (Erk2) antibodies and monoclonal antibodies raised against tyrosine-phosphorylated Erk2 (PY-Erk2), respectively. In two experiments, the PVDF membrane was treated with anti-MAP kinase (Erk2) antibodies and then reprobed with anti-tyrosine-phosphorylated Erk2 (PY-Erk2) antibodies. The next two experiments were performed using the same antibodies in opposite order. Immunoblotting with anti-Erk2 antibody showed that this protein was expressed in both HL-60 proliferating cells and in cells at all stages of differentiation (fig. 3A). Tyrosine phosphorylation of Erk2 (PY-Erk2) was detectable in proliferating HL-60 cells and increased during the first hour of differentiation (fig. 3B). After 3 h of differentiation, tyrosine phosphorylation of Erk2 decreased slightly, then gradually increased again from 12 h and reached its highest level at 48–120 h of differentiation. Thus, the

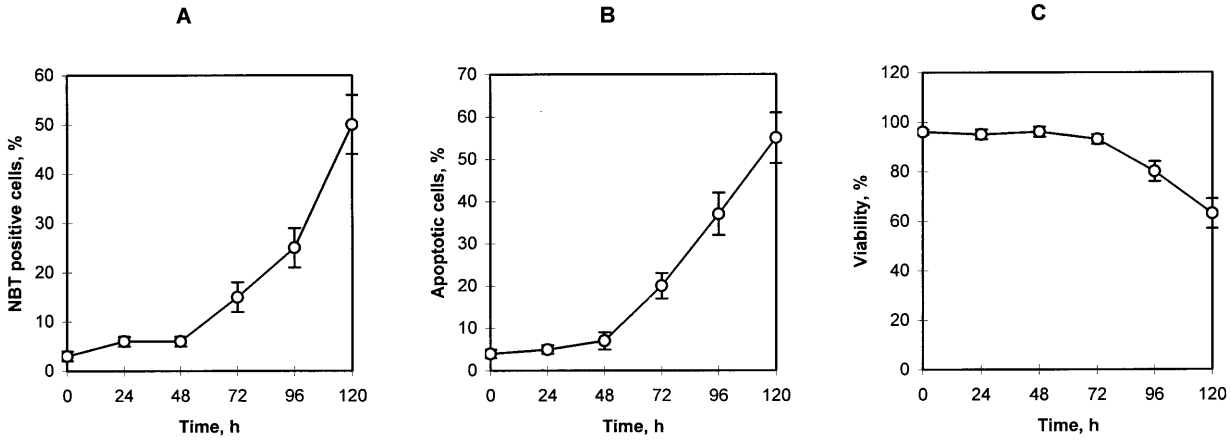


Figure 1. HL-60 cell differentiation and viability after treatment with retinoic acid (RA). HL-60 cells were exposed to 700 nM all-*trans* RA and tested for the ability of mature granulocytes to reduce nitroblue tetrazolium (*A*). The level of apoptotic HL-60 cells during cell differentiation was indicated by staining with acridine orange and ethidium bromide (*B*), and viability was estimated after staining with trypan blue (*C*). Each point represents the mean and standard deviation of three separate experiments.

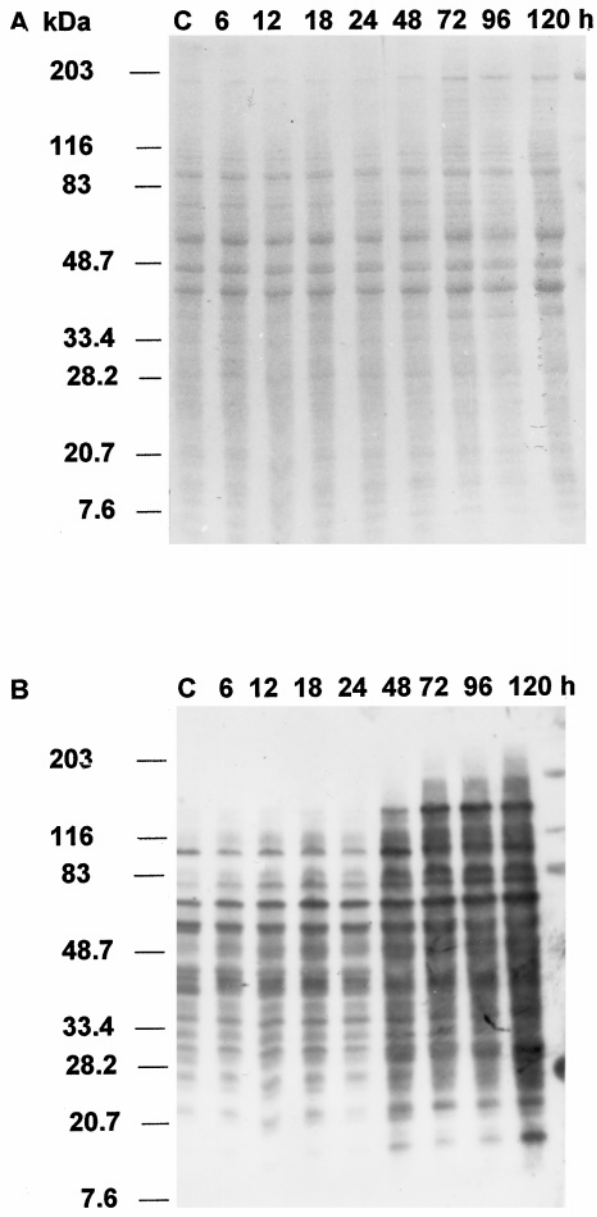


Figure 2. Electrophoretic patterns of total and tyrosine-phosphorylated cytosolic proteins during HL-60 cell differentiation. HL-60 cells were treated with 700 nM all-*trans* RA for the times indicated. Cytoplasmic proteins were prepared and subjected to SDS/PAGE electrophoresis in 7–15% acrylamide gradients. The gels were stained with colloidal CBB G-250 (*A*), or proteins were transferred onto PVDF membrane, immunoblotted with anti-phosphotyrosine antibodies, and developed by an ECL system (*B*).

high level of Erk2 tyrosine phosphorylation appeared to be a specific trait of the terminal stages during granulocyte phenotype formation, since tyrosine phosphorylation of Erk2 immediately after induction of differentiation was incomparably lower.

This last observation was provided by data from immunoblots of two-dimensional electrophoresis gels with anti-tyrosine-phosphorylated antibodies. The location of Erk2 in two-dimensional electrophoretic patterns was estimated by immunoblotting with anti-tyrosine-phosphorylated Erk2 (data not shown). As can be seen from figure 4, there is a weak signal for PY-Erk2 in control cells and in cells at precommitment stage (6–24 h) of differentiation, while at 48–96 h of differentiation, the intensity of tyrosine phosphorylation of Erk2 is much higher. It should be noted that the signal for tyrosine-phosphorylated Erk2 in mature blood neutrophils and in etoposide-induced apoptotic cells is negligible.

**Two-dimensional electrophoretic patterns of cytoplasmic proteins in proliferating, differentiated, and mature blood neutrophils.** Cytosolic proteins of proliferating, 96-h differentiated HL-60 cells and mature blood neutrophils were resolved with two-dimensional electrophoresis. More than 300 polypeptides were detected with silver staining. When loaded with proteins of the same differentiation stage, the patterns were highly reproducible. Analysis of cytoplasmic protein reference maps of control and differentiating HL-60 cells revealed that more than 30 new protein spots appeared and the relative amount of 10 proteins vanished or markedly diminished after induction of differentiation.

As can be seen in figure 5, the main differences between relative amount and number of cytoplasmic proteins of control (fig. 5A) and differentiated cells (fig. 5B) were detected for proteins with acidic and neutral pI. The spots representing newly synthesized proteins with acidic pI and apparent molecular mass of 57–66 kDa (pI 4.5–5), 40–110 kDa (pI 5.2–5.3), and 52–57 kDa (pI 5.3–5.6) are marked with semicircles (fig. 5Ba). Proteins with neutral pI can be divided into two main groups. The first possesses newly synthesized cytoplasmic proteins with an apparent molecular mass 64–80 kDa (fig. 5Bb; new spots are marked with semicircles). The second group consists of proteins with a molecular

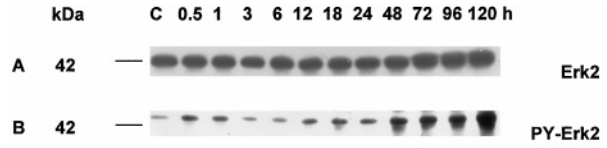


Figure 3. Western analysis of total MAPK and active Erk2 after induction of HL-60 cell differentiation. HL-60 cells were treated with 700 nM all-*trans* RA for the times indicated. Cytoplasmic proteins were prepared and subjected to SDS/PAGE electrophoresis in 7–15% acrylamide gradients. Proteins were transferred onto PVDF membrane and immunoblotted with anti-MAPK antibodies and developed by an ECL system. The same membrane was reprobed with antibodies specific to active Erk2 (PY-Erk2).

mass of 57–60 kDa. The relative amount of these proteins increased during differentiation (fig. 5Bb; new spots are marked by semicircles). Similar changes were observed in the group of cytosolic proteins with molecular weights 28.2–48.7 kDa and pI 5.7–7 (fig. 5Bc; new spots are marked by semicircles).

The relative amount of some proteins diminished and some proteins vanished completely during differentiation. Five proteins of proliferating cells with neutral pI and an apparent molecular mass of 64 kDa (pI 6.4) and 30–38 kDa (pI 6.3–6.8) were not detected after 96 h differentiation (fig. 5Bb,c; marked by open circles). The relative amount of another five proteins diminished markedly during differentiation (fig. 5Aa,c, 5Ba,c; marked by arrows).

Nine proteins, synthesized after induction of HL-60 cell differentiation, remained at the same levels in the cytoplasm of mature blood neutrophils (fig. 5Ca,c; marked by semicircles). Qualitatively, around 60 proteins detected in both proliferating and 96-h-differentiated HL-60 cells were absent in isolated mature neutrophils (fig. 5Ca–c; marked by open circles).

**Changes in tyrosine phosphorylation of cytosolic proteins at distinct stages of HL-60 cell differentiation.** Tyrosine phosphorylation of cytoplasmic proteins did not change significantly in differentiated HL-60 cells during the 24 h after induction of differentiation in comparison with proliferating cells. However, phosphorylation increased markedly during the commitment period (48–72 h) and stayed at a high level in the maturation stage (96–120 h) (fig. 2B). For a more detailed analysis of this phenomenon, cytoplasmic proteins isolated from control cells, HL-60 cells differentiated for 6, 24, 48, 72, and 96 h, human blood neutrophils, and etoposide-induced

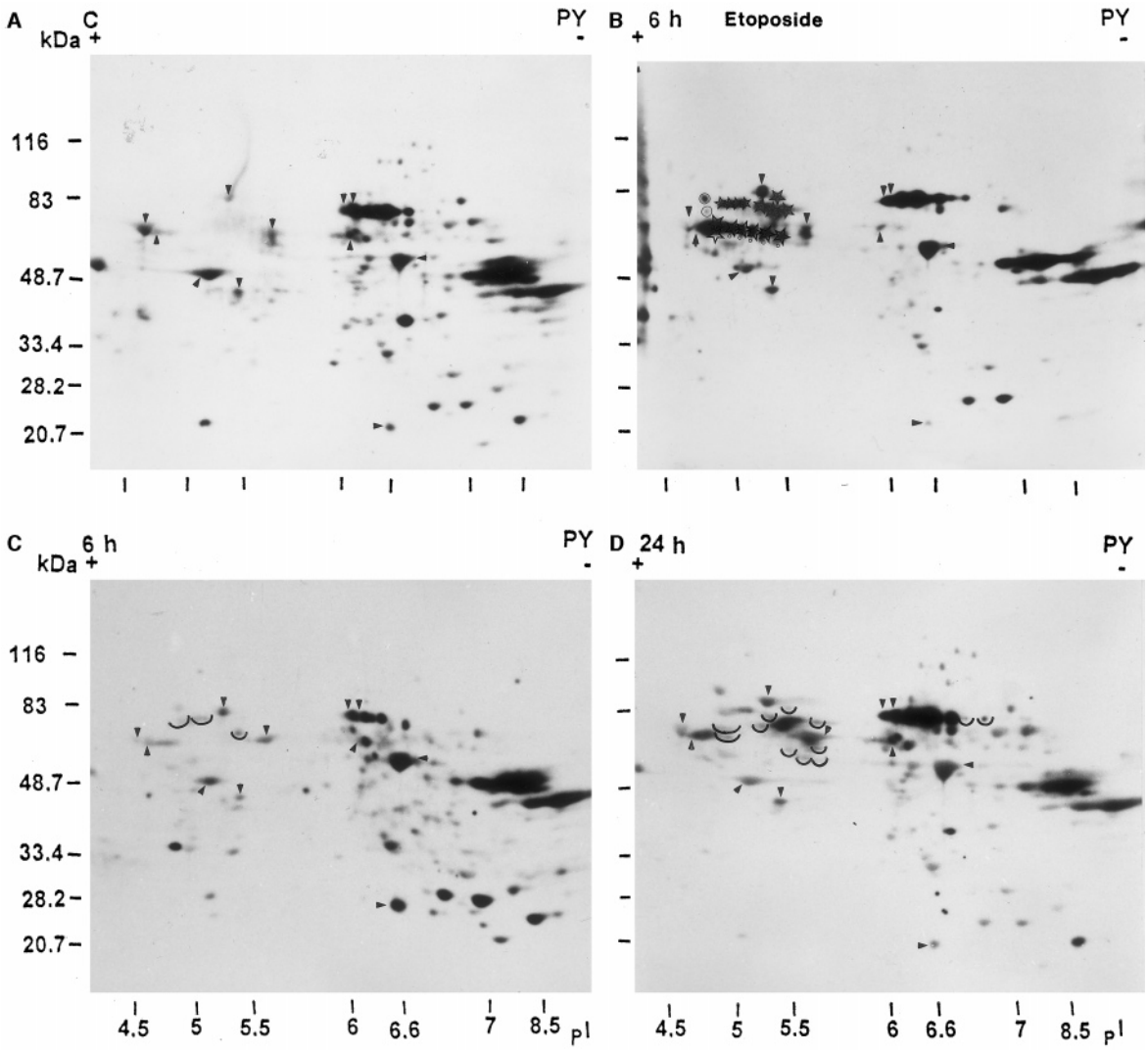


Fig. 4.

apoptotic HL-60 cells were analyzed. The proteins were fractionated using two-dimensional electrophoresis and immunoblotted with anti-phosphotyrosine antibodies (fig. 4). A high qualitative and quantitative reproducibility of tyrosine-phosphorylated protein patterns was obtained at the distinct stages of HL-60 cell differentiation. There were three types of tyrosine-phosphorylated proteins of HL-60 differentiating cells (fig. 4): proteins phosphotyrosine modified in proliferating, differentiating HL-60 cells and in mature blood neutrophils (marked by arrows); tyrosine-phosphorylated newly proteins during differentiation (marked by semicircles at the time when they first became newly tyrosine-phosphorylated); tyrosine-phosphorylated proteins of apoptotic cell origin (marked by stars).

As disclosed with two-dimensional electrophoresis (fig. 4), the intensity of tyrosine phosphorylation increased markedly at 48 h mainly for proteins with acidic pI

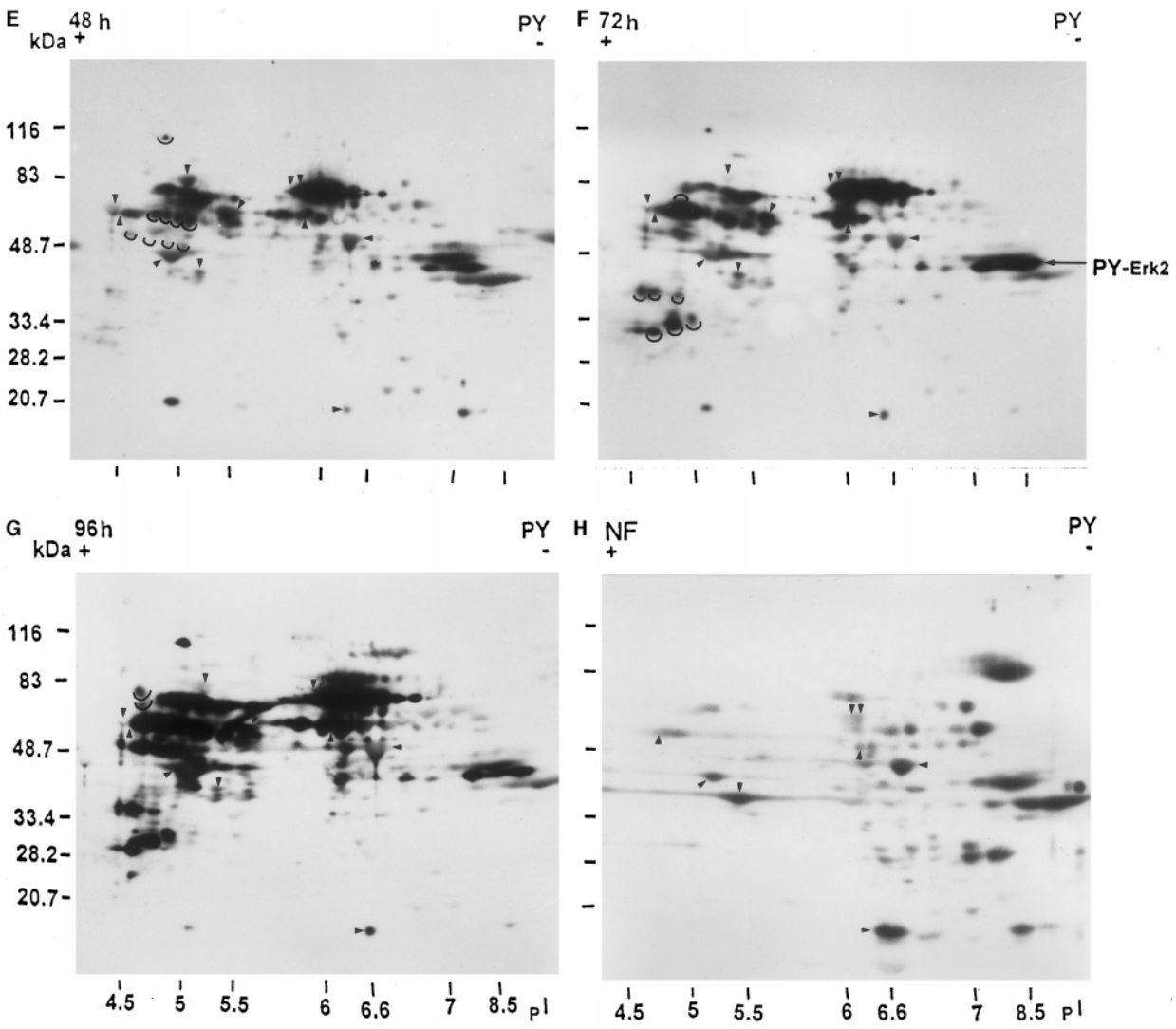


Figure 4. Changes in tyrosine phosphorylation of cytosolic proteins at distinct stages of HL-60 cell differentiation. Cytoplasmic proteins of proliferating HL-60 cells (*A*), cells treated for 6 h with 68 μM etoposide (*B*), cells differentiated with all-*trans* RA for 6 (*C*), 24 (*D*), 48 (*E*), 72 (*F*), 96 (*G*) h and mature neutrophils (NF) (*H*) were fractionated by a two-dimensional electrophoresis. Proteins were transferred onto PVDF membrane and immunoblotted with anti-phosphotyrosine antibodies. Semicircles mark new proteins modified by tyrosine phosphorylation at indicated hours. Arrowheads designate proteins permanently tyrosine-phosphorylated through all stages of differentiation and in mature neutrophils. Tyrosine-phosphorylated proteins of apoptotic cell origin, appearing at distinct stages of differentiation, are marked by different types of stars and circles. Tyrosine-phosphorylated Erk2 is specifically marked by a labelled arrow.

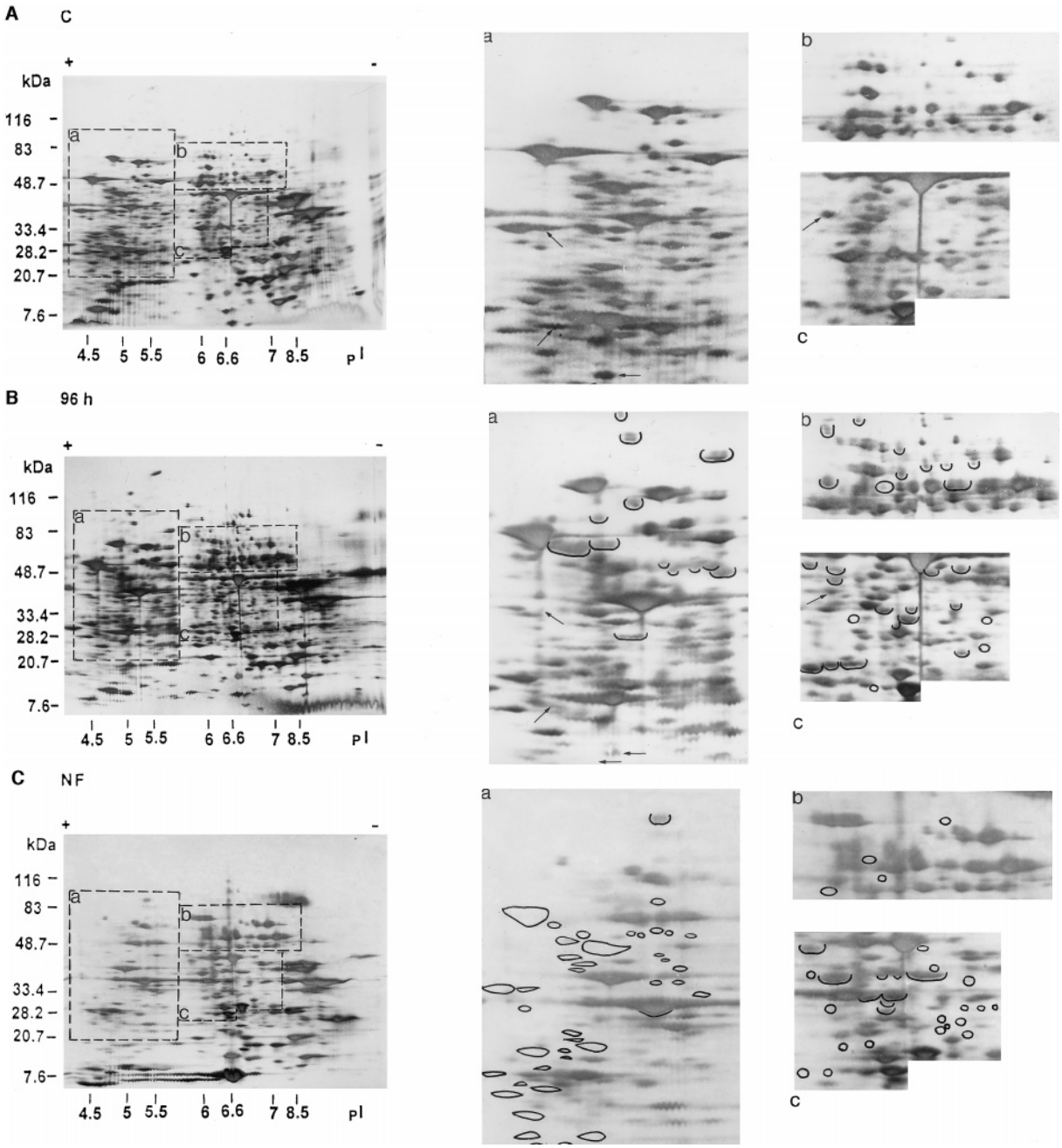


Figure 5. Two-dimensional electrophoresis of cytoplasmic proteins from proliferating and differentiated HL-60 cells and mature neutrophils. Cytoplasmic proteins of proliferating HL-60 cells (*A*), cells differentiated with all-*trans* RA (96 h) (*B*), and human blood neutrophils (NF) (*C*) were fractionated by two-dimensional electrophoresis and the gels stained with silver. Semicircles designate new proteins synthesized after induction of differentiation and in mature neutrophils in comparison with proliferating cells. Open circles mark proteins that disappeared in differentiated HL-60 cells and were absent in mature neutrophils in contrast to proliferating HL-60 cells. Arrows indicate proteins whose relative amount diminished during differentiation.

(4.5–5.5) and partially for proteins with neutral pI (6.0–7.0) and with apparent molecular masses of 45–85 kDa. At 72 h, there was intense tyrosine phosphorylation of six proteins with pI 4.5–5.0 and with an apparent molecular mass of 30–36 kDa. By contrast, the intensity of tyrosine phosphorylation of some proteins in the pI range 6.6–8.5 with apparent molecular masses of 20–33 kDa decreased.

Although the highest percentage of differentiated cells in the population was observed at 96–120 h, cell viability decreased from 80 to 65% (fig. 1C) and a large proportion (35–55%) of apoptotic cells appeared (fig. 1B). It should be pointed out that the population of HL-60 cells treated with etoposide for 6 h also contained a large number (approximately 80%) of apoptotic cells. Tyrosine phosphorylation of a few 60- to 80-kDa proteins with pI 4.5–5.5 increased markedly during the maturation stage of differentiation and also in HL-60 cells treated for 6 h with etoposide (fig. 4B; marked by stars). This finding suggests that the above-mentioned proteins are involved in an apoptotic process. This suggestion is strongly confirmed by the absence of these tyrosine-phosphorylated proteins in two-dimensional electrophoretic patterns of both proliferating cells and cells differentiated for 6–24 h (containing 3 and 5% apoptotic cells, respectively).

## Discussion

In the present study, we clearly showed that the total tyrosine phosphorylation level of cytoplasmic proteins increases significantly at the time of commitment and at the terminal stages of HL-60 cell differentiation (48–120 h) induced by RA. The fraction of differentiated cells in the population increased by up to 50–55% after 120 h (fig. 1A). Distinct patterns of proteins and different degrees of their tyrosine phosphorylation were seen during proliferation and at characteristic stages of RA-induced differentiation. There were particularly large differences in the phosphotyrosine content in terminally differentiated HL-60 cells. The latter observation suggests that changes in long-term tyrosine phosphorylation of cytosolic proteins in differentiating HL-60 cells are associated with the formation of the mature granulocyte phenotype. This is supported by the observations of other investigators [26, 53] suggesting that differentiation is associated with activation of tyrosine kinases. Indeed, two tyrosine protein kinase activities ($p93^{c\text{-}fes}$ and $p60^{src}$) have been identified in differentiated HL-60 leukemia cells [54, 55], and a protein tyrosine kinase related to the FER gene product has been distinguished in HeLa cells [56]. On the other hand, we found a number of tyrosine-phosphorylated proteins highly expressed in differentiated HL-60 cells but absent in mature human blood neutrophils.

The level of apoptotic cells during HL-60 cell differentiation (fig. 1B) started to increase from the commitment stage (48 h) and reached around 50% at the terminal stage (120 h) of differentiation. The relationship between cellular differentiation and apoptosis in the context of RA activity is still unclear and published results are conflicting. In the human erythroleukemia cell line HEL, RA has been shown to induce differentiation without indications of apoptosis [57]. According to others [6, 7], a significant fraction of HL-60 cells become apoptotic upon treatment with RA, and in acute promyelocytic leukemia cells, RA down-regulates the expression of the oncogene Bcl2, which prevents neoplastic cells from entering into the apoptotic pathway. Recently, Carpentier et al. [8] showed that RA alone could not induce apoptosis, but did so in conjunction with co-factors such as fetal calf serum proteins, glucose, and low pH. The depletion of some components of the medium and the appearance of secreted multiple factors could have influenced the induction of apoptosis.

The molecular effectors of the programmed cell death pathway are present in all cell types, and they can probably all be induced to die by apoptosis because the mitotic and apoptotic pathways have common effector molecules [58, 59]. Multiple kinase cascades and regulatory mechanisms initiate the entry into the mitotic or differentiation programs [38]. Correlations between tyrosine kinase activation [39–42], or inhibition [43–48], and enhancement of apoptosis have been observed in various experimental systems. Radiation-induced apoptosis in B lymphocyte precursors required the activation of tyrosine kinases [39]. Usami et al. [40] showed that pretreatment of U937 cells with a potent tyrosine kinase inhibitor significantly suppressed the appearance of characteristic apoptotic morphological changes. Specific tyrosine phosphorylation of several proteins following the stimulation of thymocytes in a human T cell line [41] and during Fas-mediated apoptosis has been demonstrated [42]. In contrast, inhibition of apoptosis after activation of tyrosine kinases has been shown in HL-60 cells [43, 48], cultured ovarian granulosa cells [44], cells transformed with Abl or Bcr/Abl [45, 47], and in eosinophils and neutrophils [46]. Bruton's tyrosine kinase (BTK) has a dual function in regulation of apoptosis in B cell precursors. BTK promotes radiation-induced apoptosis but inhibits Fas-activated apoptosis [49].

Other indirect evidence suggests that RA can modulate apoptosis by stimulating protein kinase C (PKC) [60]. Indeed, PKC activity has been reported to be involved in apoptosis in the myeloid leukemia cell lines HL-60 and P39 [61, 62]. Fine-tuning of phosphorylation is apparently important, since inhibitors of serine/threonine phosphatases affect the susceptibility to apoptosis in a variety of cell types—cultured leukemia cells [63], retinoblastoma cells [64], T cells [65], and breast cell lines [66]. Protein tyrosine phosphatase activity affects FAS-induced cell death [67], and in hematopoetic cells, it appears to be required for optimal FAS-mediated apoptosis [68].

We reported here an increase in PY-Erk2 activity accompanying RA-induced differentiation. Erk2 activation by MAPK/Erk kinase (MEK) was recently shown to be crucial for RA-induced differentiation of HL-60 cells [69]. In the case of MEK/ERK kinase cascades, phosphorylation or dephosphorylation of transcription and translation factors may determine whether a cell will divide, arrest in $G_0/G_1$, or die by apoptosis [70]. Activation of JNK and p38MAPK and concurrent inhibition of ERK contributes to cell death in rat PC12 cells [71]. p38MAPK and JNK are implicated in the pathways leading to stress-induced apoptosis in human neutrophils [72] and amplify the apoptotic response in some other cell lines [73]. MEK kinase 1 is also a strong stimulator of apoptosis [74], but the signalling patterns are confusing. ERK promotes cell survival in nerve growth factor-differentiated PC12 cells [71] and myeloid leukemia cells [70, 75], and PMA-induced MEK/ERK activity regulates cell cycle arrest in differentiating K562 [76] and HL-60 [77] cells. Our results suggest that a significant increase in the phosphotyrosine content of Erk2 at the commitment and terminal stages of HL-60 cell differentiation (48–120 h) is related to promotion of cell survival or triggering of apoptosis. A very recent observation [78] suggests that Erk2 is the target of hydrogen peroxide ($H_2O_2$). This is interesting in view of our results demonstrating increased activation of Erk2 in HL-60 cells maturing to become NBT positive and granulocyte-like with a capacity to generate an oxidative burst ($H_2O_2$).

In this study we demonstrated that the program of phenotype formation entails dramatic changes in long-term tyrosine phosphorylation of some cytoplasmic proteins during RA-induced HL-60 cell differentiation to granulocytes. We have also shown that a high level of Erk2 tyrosine phosphorylation is a characteristic feature for granulocyte maturation mediated by RA. In addition, we were able to elucidate some cytosolic proteins of apoptotic origin. The patterns of tyrosine phosphorylation of specific cytosolic proteins in maturing and apoptotic granulocytes should be powerful tools for further elucidation of differentiation mechanisms.

Two inducers of terminal granulocytic differentiation (RA and DMSO) have been shown to induce myeloid differentiation along two distinct pathways [79] and three monocytic inducers did not induce the same metabolic cascade leading to differentiation [80]. Therefore, to discriminate specific proteins for distinct signalling pathways in the same cell lineage and specific proteins for a myeloid or monocytic lineage, we should compare the patterns of tyrosine-phosphorylated cytoplasmic proteins isolated from control and differentiating HL-60 cells: (i) induced to differentiate to granulocytes by RA (activation of the PKC signalling pathway and nuclear targeting of the heterodimer of the retinoic acid receptor—RAR-RXR) and by $N^6$,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (activation of the adenylyl cyclase signalling pathway); (ii) induced to differentiate to monocytes by PMA (activation of the PKC signalling system) and by 1,25-dihydroxyvitamin $D_3$ (targeting the heterodimer of the 1,25-dihydroxyvitamin $D_3$ nuclear receptor—VDR-RXR); (iii) induced to differentiate to granulocytes by RA and to monocytes by 1,25-dihydroxyvitamin $D_3$ [1, 5, 81–84].

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