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Research Article

Long-term changes in tyrosine phosphorylation of the abundant nuclear proteins during granulocytic differentiation of HL-60 cells

A. Gineitis[a,*], G. Treigyte[a], J. Savickiene[a], V. P. Shanbhag[b] and T. Stigbrand[b]

[a]Laboratory of Developmental Biology, Institute of Biochemistry, Vilnius (Lithuania)
[b]Departments of Biochemistry and Immunology, University of Umeå, S-90185 Umeå (Sweden)

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Abstract. The two-dimensional electrophoretic patterns of nuclear proteins and their tyrosine phosphorylation were compared for HL-60 cells before and after differentiation induction to granulocytes by dimethyl sulfoxide, all-trans retinoic acid and N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate. Regardless of the inducer used, some nuclear proteins, which are tyrosine-phosphorylated in proliferating HL-60 cells, undergo gradual dephosphorylation 12–72 h after induction of differentiation, followed by drastic dephosphorylation during maturation to granulocytes. At least 13 nuclear proteins with a molecular mass of 35–110 kDa are dephosphorylated, and 6 nuclear proteins undergo tyrosine phosphorylation. Analysis of the nuclear proteins differentially extracted by salt and detergents indicates that changes in their tyrosine phosphorylation during the maturation stage of differentiating granulocytes occur mainly in proteins which are abundant in nucleoplasm, chromatin and residual nuclear structures. The abundance of these proteins, residing in the nuclear structures, and their long-term modification in phosphorylation during the maturation stages of differentiation strongly suggest that tyrosine phosphorylation of these proteins is involved in reorganization of the differentiating cell nucleus.

Key words. Leukocytes; maturation.

The human promyelocytic leukemia cell line HL-60 can be induced to differentiate to mature granulocyte-like cells by dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA) and N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate (dbcAMP) [1]. These compounds induce differentiation through receptors that are localized in different compartments of the cell and activate different second messenger systems. DMSO stimulates protein kinase C activity [2], dbcAMP stimulates the adenylyl cyclase system [3] and RA activates the retinoic acid receptor, a transcription factor located in the nucleus [4]. Regardless, all three inducers cause differentiation to the same granulocytic phenotype. Therefore, the possibility exists that these three distinct signaling pathways leading to the same pattern of gene expression in terminally differentiated cells should finally converge during the commitment period of differentiation or later to one common gene regulatory mechanism in the nucleus in which tyrosine phosphorylation-dephosphorylation is involved.

Protein tyrosine phosphorylation is one of the events by which cell proliferation, differentiation and metabolism are regulated by external stimuli. It was shown that cytoplasm-signaling proteins which are targets for lig-

* Corresponding author. Current address: Department of Biological Chemistry, Tupper Hall, School of Medicine, UC Davis (California 95616, USA), Fax + 1 530 752 3516.

and-activated receptor tyrosine kinases or nonreceptor tyrosine kinases share a common structural element, the src homology (SH2, SH3) domains, which mediate the phosphotyrosine kinase-substrate interaction. Tyrosine-phosphorylated signaling proteins activate Ras, which is a crucial mediator in many biological responses [5]. Some signaling proteins are transcription factors, like the members of the STAT (signal transducers and activators of transcription) family and mitogen-activated protein (MAP)-kinases, which after tyrosine phosphorylation in the cytoplasm are translocated to the nucleus [6–11]. Tyrosine kinases—Abl, Fer, Fes, Rak, Lyn, Wee1—and MAP kinase have been observed in the nucleus [12–20]. The rapid inactivation of nuclear tyrosine phosphorylated STAT and MAPK depends on tyrosine phosphatase or MAP-kinase phosphatase [21–23]. There are also some protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) which show specificity for the nuclei of haematopoietic cells [15, 16, 24]. It has been shown that PTPs demonstrate some homology with the basic domain of transcription factors and bind chromatin or DNA [25–28]. PTKs and PTPs are involved in the regulation of the timing of mitosis [29–31], and they modulate the activity of the tumor suppressor proteins pRb and p53 [17, 32]. There are several examples involving tyrosine phosphorylation of nuclear proteins after cell activation. Active chromatin isolated from rodent kidney nuclei shows PTK activity in both exogenous and endogenous nuclear substrates [33]. Growth hormone rapidly stimulates the tyrosine phosphorylation of at least eight nuclear proteins [34]. In addition, unlike normal fibroblasts, the nuclei of fibroblasts transformed by v-*abl* contain some tyrosine-phosphorylated proteins [35]. A protooncogene, p80 c-rel, belonging to a family of transcription factors which display cytoplasmic-nuclear partitioning, is rapidly tyrosine-phosphorylated following granulocyte colony-stimulating factor treatment of human neutrophils [36]. Phosphorylation of tyrosine modulates mammalian RNA polymerase II interaction with the preinitiation complex in HeLa cells [37]. The correlation between tyrosine dephosphorylation, increased binding of a transcriptional complex and stimulation of gene expression by transforming growth factor (TGF)β1 has been observed recently [38]. It has been shown that PTK and PTP activities increase and the phosphotyrosine content significantly decreases along with HL-60 cell differentiation to both the granulocytic and monocytic phenotypes [39, 40]. However, in these studies data concerning distinct protein tyrosine phosphorylation were not presented.

These findings prompted us to investigate tyrosine phosphorylation of nuclear proteins during HL-60 cell differentiation to granulocytes mediated by DMSO, RA and dbcAMP. We have shown in this study that about 20 nuclear proteins with a molecular mass in the region of 35–110 kDa are tyrosine-phosphorylated or dephosphorylated during the final stages of HL-60 cell differentiation.

Materials and methods

Chemicals and solvents. DMSO, RA, dbcAMP, nitro blue tetrazolium (NBT), phorbol 12-myristate 13-acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin, NaF, sodium orthovanadate (Na_3VO_4), DNase I, RNase A, Tris, EDTA, SDS, Nonidet P-40, Tween-20, dithiothreitol (DTT), 2-mercaptoethanol, glycine, sucrose, urea and glycerol were obtained from Sigma. Acrylamide, methylene-bis-acrylamide (BIS), *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) and Coomassie Brilliant Blue G-250 (CBB G-250) were from Bio-Rad. Cell culture medium RPMI 1640, fetal bovine serum, penicillin and streptomycin were from Gibco. Protein molecular mass markers and Ampholines pH 3.5–10, 6–8, 7–9 and 9–11 were from Pharmacia. Peroxidase-conjugated rabbit anti-immunoglobulin (Ig) to mouse Ig was purchased from DAKOPAT, anti-phosphotyrosine antibodies PY-20 were from ICN Biochemicals and the enhanced chemiluminescence Western blot detection system (ECL) was from Amersham. Other chemicals used in this work were of analytical grade.

Culture conditions. HL-60 promyelocytic leukemia cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Cells were seeded at a level of 4×10^5 cells/ml and were allowed to attain a maximum density of 1.5×10^6 cells/ml before being transferred to a fresh medium. Passaged cells were not used beyond passage number 60. Granulocytic differentiation was induced in HL-60 cells by treatment with 1.3% DMSO or 250 μM dbcAMP or 500 nM RA. Stock solutions of 200 μM RA, in 96% ethanol and 10 mM dbcAMP in deionized water, stored at −20 °C, were used. Differentiation was induced at a cell concentration of 5×10^5 cells/ml. The extent of differentiation was assayed by the ability of the cells to reduce NBT to insoluble blue-black formazan on stimulation by PMA [41]. Cell suspension (100 μl) from the cultures was mixed with an equal volume of 0.2% NBT dissolved in phosphate-buffered saline containing 40 ng/ml of PMA and incubated at 37 °C for 30 min. NBT-positive cells were counted using a haemocytometer. At least 200 cells were scored for each determination, and the number of cells positive for NBT reductivity was expressed as percentage of the total viable cell number, determined by the exclusion of 0.2% trypan blue. The differentiating populations containing no less than 75–80% differentiated cells were used.

Preparation of nuclei. Cells were collected, washed twice in 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1 M NaCl, pH 7.5 (PBS) containing 10 mM NaF and 1 mM Na_3VO_4, resuspended at a level of 3×10^7 cells/ml in solution A (10 mM NaCl, 10 mM Tris/HCl, pH 7.5, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM PMSF, 10 μg/ml of pepstatin, 10 μg/ml of leupeptin, 10 mM NaF, 1 mM Na_3VO_4) and kept at 0 °C for 15 min to swell [42]. Then 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 740*g* for 5 min. The supernatant (cytosol fraction) was clarified by centrifugation at 15000*g* for 15 min and frozen at − 70 °C. The pelleted nuclei were washed twice with a mixture of solution A and B (1:1) and three times by centrifugation at 740*g* for 5 min with solution C (10 mM NaCl, 10 mM Tris/HCl, pH 7.5, 3 mM $MgCl_2$, 2 mM DTT, 0.3 M sucrose, 1 mM PMSF, 10 μg/ml of pepstatin, 10 μg/ml of leupeptin, 10 mM NaF and 1 mM Na_3VO_4). The purity and integrity of washed nuclei were checked under a light microscope and then frozen at − 70 °C.

Nuclear protein fractionation. Nuclear protein fractions were obtained by sequential extraction of the nuclear pellet with 0.15 M NaCl and 0.35 M NaCl in 10 mM Tris/HCl buffer, pH 7.5 containing 2 mM EDTA. In each case, the nuclei were extracted twice by 5 volumes of the saline-buffered solution for 30 min at 0 °C and washed four times with large volumes of the same solution by centrifugation at 1500*g* for 15 min. The nucleoplasm fraction extracted by 0.15 M NaCl, and the nonhistone chromatin proteins extracted by 0.35 M NaCl, as well as the residual nuclear pellet were used immediately for electrophoresis or were frozen at − 70 °C.

Preparation of samples for electrophoresis. To prepare samples for SDS-polyacrylamide gel electrophoresis (PAGE) 12.5 μl of 0.5 M Tris/HCl, pH 6.8, 30 μl of 10% SDS, 20 μl of 0.5 M DTT and 20 μl of glycerol were added to 100 μl of the nuclear protein fraction or to 100 μl of the suspension of the residual nuclei in deionized H_2O, and the mixtures were boiled for 5 min. Electrophoresis samples of total nuclear proteins for nonequilibrium pH gradient electrophoresis (NEPHGE) were prepared as follows: approximately 5×10^7 nuclei/ml were suspended in 240 μl of solution A (10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM DTT), 20 μl of solution B (0.5% SDS, 10 mM DTT) and 50 μl of solution C (DNAse I 2 mg/ml, RNAse 1 mg/ml in 10 mM $MgCl_2$ and 20 mM Tris/HCl, pH 7.4) and incubated at 0 °C for 1 h. Then 288 mg of urea and 240 μl of solution D (0.25% SDS, 3% Nonidet P-40, 100 mM DTT, 2% Ampholine pH 6–8, 2% Ampholine pH 7–9 and 2% Ampholine pH 3.5–10) were added. The samples were centrifuged at 15000*g* for 15 min before use.

2D electrophoresis. Two-dimensional (2D) NEPHGE-SDS-PAGE was performed according to a method similar to that of O'Farrell [43]. The first dimension was carried out on a cylindrical electrofocusing gel (2.5 mm × 80 mm) prepared as follows: 2.75 g of urea was added to 0.5 ml of a solution of 38% acrylamide, 2% BIS, 1 ml of Nonidet P-40, 1.2 ml of H_2O, 65 μl of Ampholine pH 6–8, 65 μl of Ampholine pH 7–9 and 125 μl of Ampholine pH 9–11. Electrofocusing was run for 4 h at 500 V. For the second dimension, gels were equilibrated for 1 h in a solution containing 10% glycerol, 50 mM DTT, 2.3% SDS, 625 mM Tris/HCl, pH 6.8 and 0.001% bromophenol blue. The first dimension gel was then tightly fixed at the top of the second dimension gel and covered by a 1-mm layer of equilibration solution. Agarose was not used for gel fixing. The second dimension electrophoresis was carried out on a 1.5-mm thick, pore gradient gel (160 mm × 140 mm or 85 mm × 55 mm) composed of 7.5–15% acrylamide containing 0.1% SDS using a current of 1 mA/mm^2 until the bromophenol blue had left the gel. The gel was then stained using the nondiamine silver-staining method [44] or with colloidal CBB G-250 [45].

Immunoblotting with anti-phosphotyrosine antibodies. To transfer proteins to the PVDF membrane (Bio-Rad), the gel was incubated for 1 h in transfer buffer (12.5 mM Tris, 96 mM glycine, 0.181% SDS, 10% methanol), and proteins were transferred using the Trans blot cell (Bio-Rad) and a voltage of 60 V for 2 h. The membrane was blocked with 2.5% BSA dissolved in PBS-Tween 20 (PBS solution containing 0.05% Tween 20) overnight at 0 °C and washed five times with PBS-Tween 20′ (PBS solution containing 0.12% Tween 20). The membrane was then incubated at room temperature for 1 h with 1:6000 dilution of anti-phosphotyrosine antibodies PY-20 in PBS-Tween 20′, washed five times during 30 min with PBS-Tween 20′, then incubated further with 1:2000 dilution of peroxidase-conjugated rabbit Ig to mouse IgG antibodies in PBS-Tween 20′ for 1 h at room temperature and washed again in the same manner as above. Immunoreactive proteins were visualized using ECL Western blotting reagents.

Results

Tyrosine phosphorylation of nuclear proteins during HL-60 cell differentiation

Proteins were fractionated by SDS electrophoresis in a 7.5–15% gradient polyacrylamide gel into at least 50 bands (fig. 1a). The comparison of electrophoretic patterns reveals that there are insignificant quantitative differences between visible nuclear proteins of control and differentiating cells. Figure 1a shows that in differentiating cells the relative intensities of four bands with

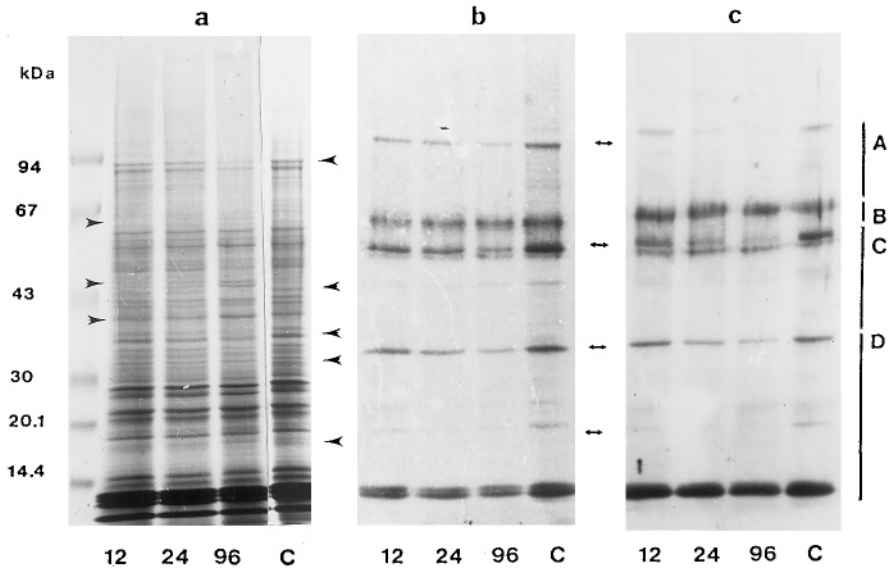

Figure 1. Time course changes in the relative amount and tyrosine phosphorylation of nuclear proteins during HL-60 cell differentiation. Differentiation was induced by adding (*a*, *b*) 1.3% DMSO (v/v) or (*c*) 500 nM RA to HL-60 cell culture (5×10^5 cells/ml). 12, 24, 96, time in hours after induction of differentiation; C, control cells. Nuclei were isolated from differentiating cells at different time points after induction of differentiation or from control cells, and the total nuclear protein samples for SDS-PAGE in a 7.5–15% gradient polyacrylamide gel were prepared as described in 'Materials and methods'. The proteins of DMSO-induced cells were visualized following staining by Coomassie Brilliant Blue G-250 (*a*); the proteins of DMSO (*b*) and RA-induced cells (*c*) were immunoblotted with anti-phosphotyrosine antibodies. The proteins of DMSO- and RA-induced cells were run on the same gel (stained protein pattern of RA-induced cells not shown) and immunoblotted on the same polyvinylidene difluoride (PVDF) membrane. The proteins that change in relative amount during differentiation are indicated in (*a*) by arrows: for the proteins increasing in amount at the left and for the proteins decreasing in amount at the right. The proteins in which tyrosine phosphorylation decreases during differentiation are indicated by double arrows.

apparent molecular mass of 40, 48–50 and 64 kDa increased, while those of the other five bands with apparent molecular mass of 90, 44, 38, 32 and 18 kDa decreased, and the time of this change (96 h) coincides with myeloid cell maturation.

Figure 1 represents Coomassie Brilliant Blue G-250 stained (*a*) and immunoblotted with anti-phosphotyrosine antibodies (*b*, *c*) patterns of the total nuclear proteins isolated from differentiating cells at different times, starting 12 h after induction with DMSO, RA and from control cells. Stained (*a*) and immunoblotted (*b*, *c*) proteins were run on the same gel. We have not found any marked changes in protein content and degree of tyrosine phosphorylation during the early periods (0.5–6 h) after induction with the three inducers used (results are not shown). Some changes in the relative amounts of distinct proteins during differentiation have been mentioned above (fig. 1a). There are at least 15 visible tyrosine-phosphorylated protein bands in the total protein pattern of uninduced HL-60 cells (fig. 1b), and four give a high phosphotyrosine signal. All minor bands containing tyrosine-phosphorylated proteins with a molecular mass between those of the major bands marked A, B, C and D are assumed to belong to the group with the higher molecular weight. Thus, group A consists at least of five bands, group B of two bands, group C of five bands and group D of three bands. There are two bands with molecular mass less than 14 kDa carrying a high tyrosine phosphorylation signal whose localization coincides with the electrophoretic mobility of histones H3 and H2B. An extremely high relative amount of four histones in the nuclear protein fraction might be the reason for nonspecific interaction of anti-phosphotyrosine antibodies with phosphorylated serine or threonine in histone molecules. It should be noted that the relative amounts of proteins with electrophoretic mobility of Coomassie Brilliant Blue-stained bands (fig. 1a) coinciding with four major phosphotyrosine signals (bands A, B, C and D) in figure 1b,c are between one (bands C and D) to two (bands A and B) orders lower than the amounts of histones. Such a generalized pattern of changes in relative protein content and degree of tyrosine-phosphorylated proteins is typical for all cases of HL-60 differentiation, regardless of the inducer used (DMSO and RA in fig. 1 and dbcAMP in fig. 2). The majority of

these proteins are dephosphorylated gradually during the process of differentiation with a more prominent decrease of phosphorylation at the maturation stage (96–120 h after induction) of differentiation. The time course changes in tyrosine phosphorylation of nuclear proteins during dbcAMP-mediated differentiation demonstrates that some of the tyrosine-phosphorylated proteins belonging to groups A, C and D are gradually dephosphorylated during 12–72 h and dramatically dephosphorylated 96–120 h after induction of differentiation (fig. 2a). It should be pointed out that the extent of dephosphorylation is proportional to the number of differentiating cells in the population (results not shown) and is maximal at 96–120 h (fig. 2) after induction, when the number of differentiated cells no longer changes. These data suggest that there is no gradual dephosphorylation in individual cells, but the pattern is characteristic for a growing or differentiating cell population. With this in mind, nuclear proteins isolated from growing control cell populations and populations with a maximal number (75–80%) of differentiated cells were fractionated by 2D gel electrophoresis and immunoblotted with anti-phosphotyrosine antibodies for a more detailed examination of tyrosine phosphorylation (fig. 3). On the 2D gel stained with colloidal CBB G-250 about 70 spots can be seen (fig. 3a,b). Immunoblotting analysis of these nuclear protein patterns demonstrated that the major bands possessing a tyrosine phosphorylation signal in 1D electrophoresis (fig. 1b,c) are not homogeneous. Tyrosine-phosphorylated proteins in control cells which subsequently undergo dephosphorylation during differentiation (absent or diminished tyrosine phosphorylation signal in fig. 3d) are marked by numbers in figure 3c and proteins which undergo tyrosine phosphorylation during differentiation are marked by numbers in figure 3d. It should be noted that the tyrosine phosphorylation signal in spot 20 is equally strong in patterns from control (fig. 3c) and differentiated (fig. 3d) cells, and therefore the degree of tyrosine phosphorylation of the corresponding protein does not change during differentiation. Thus, according to the 2D gel electrophoresis data, at least 13 tyrosine-phosphorylated nuclear proteins in uninduced HL-60 cells are dephosphorylated, and at least 6 nuclear proteins undergo phosphorylation during granulocytic differentiation. It should be noted that only 11 abundant nuclear proteins with a tyrosine phosphorylation signal are visible as spots (3, 7, 8, 9, 12–15, 18, 19, 20) in the electrophoretic protein pattern of control and differentiated cells stained by Coomassie Brilliant Blue G-250 (fig. 3a,b). The relative amount of these proteins is very similar in control and differentiated cells, whereas their tyrosine phosphorylation signals differ markedly. Moreover, the 2D gel electrophoresis pattern (fig. 4) in which the total nuclear proteins of the control and the differentiated HL-60 cells are resolved into more than 200 silver-stained spots reveals that the steady-state concentrations of individual tyrosine-phosphorylated proteins do not change during differentiation.

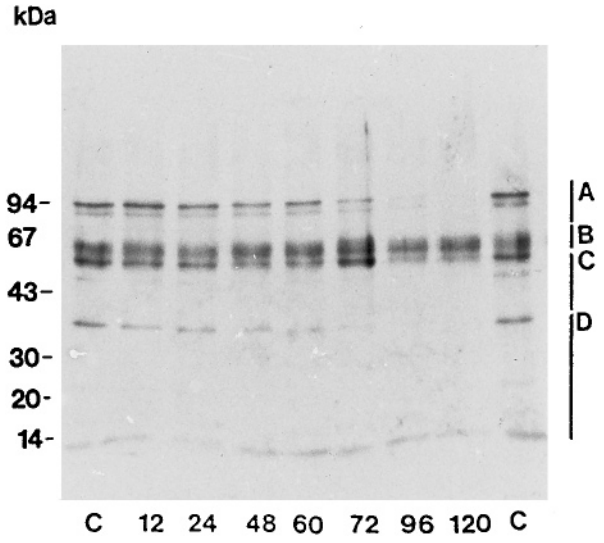

Figure 2. Time course changes in nuclear protein tyrosine phosphorylation during HL-60 cell differentiation. Differentiation was induced by adding 250 μM dbcAMP to HL-60 cell culture (5×10^5 cells/ml). Nuclei were isolated from differentiating cells at different time points after induction of differentiation or from control cells, and the total nuclear protein samples for SDS-PAGE in a 7.5–15% gradient polyacrylamide gel were prepared as described in 'Materials and methods'. After electrophoresis, proteins were immunoblotted with anti-phosphotyrosine antibodies. 12, 24, 48, 60, 72, 96, 120 denote hours after induction of differentiation; C denotes control cells.

Tyrosine phosphorylation of nucleoplasm proteins and proteins loosely and tightly bound to nuclear structures. In order to elucidate the distribution of tyrosine-phosphorylated proteins within the nuclear structures, the nuclei were sequentially extracted with solutions containing increasing concentrations of sodium chloride. It is known that nucleoplasm proteins can be extracted with 0.15 M NaCl, and nucleoprotein complexes are insoluble at this ionic strength. At a concentration of 0.35 M NaCl loosely bound nonhistone proteins dissociate from chromatin. Histones, nonhistone proteins tightly bound to DNA and nuclear matrix proteins could only be solubilized by incubation of salt-extracted nuclei in a solution containing 2.5% SDS and a disulfide bond-reducing agent [46].

These fractions of nuclear proteins isolated from control HL-60 cells were fractionated by SDS-PAGE and immunoblotted using anti-phosphotyrosine antibodies (fig. 5). It should be pointed out that at each ionic strength proteins have been taken from two initial ex-

tractions and the nuclei afterwards were extensively washed with the same salt solution. As can be seen from the pattern of immunoblotting (fig. 5), there are at least 10 tyrosine-phosphorylated proteins in the nucleoplasm, six in the 0.35 M NaCl fraction and 6 in the residual proteins of chromatin and nuclear matrix fraction. Thus, these three nuclear protein fractions contain more than 20 tyrosine-phosphorylated proteins. This number of proteins carrying a tyrosine phosphorylation signal coincides with that obtained in 2D gel electrophoresis (fig. 3). The data strongly suggest that some nucleoplasm, nonhistone and residual proteins of chromatin are tyrosine-phosphorylated in uninduced HL-60 cells, and they undergo tyrosine dephosphorylation during induced differentiation.

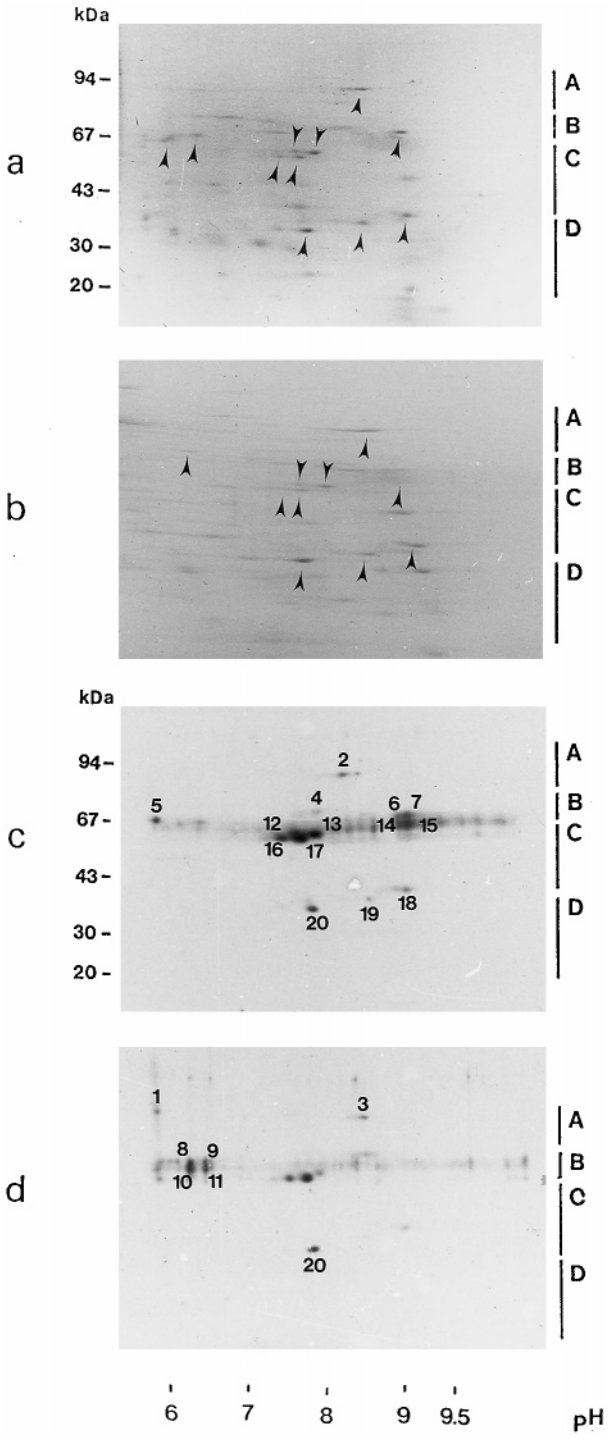

Figure 3. Two-dimensional electrophoresis and immunoblotting of nuclear proteins isolated from control and differentiated HL-60 cells. The total nuclear proteins were isolated from control and differentiated HL-60 cells 120 h after induction with 1.3% DMSO (v/v). The protein samples were prepared as described in 'Materials and methods'. The proteins were fractionated by 2D electrophoresis: NEPHGE (pH 6–9) in 5% PAG and SDS-PAGE in a 7.5–15% gradient polyacrylamide gel (core histones not shown). The proteins from both control and differentiating cells were fractionated by 2D electrophoresis and were transferred on the same PVDF membrane, immunoblotted and developed by an ECL system under identical conditions; (*a*) the proteins from control cells stained by colloidal Coomassie Brilliant Blue G-250; (*b*) the proteins from differentiated cells stained by colloidal CBB G-250; (*c*) the proteins from control cells immunoblotted with anti-phosphotyrosine antibodies; (*d*) the proteins from differentiated cells immunoblotted with anti-phosphotyrosine antibodies. Arrows in (*a*, *b*) indicate abundant nuclear proteins modified by tyrosine phosphorylation; numbers in (*c*) indicate tyrosine-dephosphorylated proteins, whereas numbers in (*d*) indicate tyrosine-phosphorylated proteins during differentiation.

Discussion

We demonstrate here for the first time that there are specific patterns of long-term tyrosine phosphorylation of nuclear proteins in growing and differentiating cells of the promyelocytic cell line HL-60. In our experiments the total number of proteins in the nuclear fraction was resolved into at least 200 spots when stained by silver and into approximately 70 spots when stained by the Coomassie Brilliant Blue G-250 stain. More than 10 proteins that are visible in the Coomassie Brilliant Blue G-250 stained pattern gave a high signal of tyrosine phosphorylation on Western blots. The time course changes in tyrosine phosphorylation of these proteins are very similar in HL-60 cells induced to differentiate by all three inducers despite the activation of different second messenger systems. The three possible reasons for this similarity in differentiation could be (i) the method used is not sensitive enough to detect subtle changes in tyrosine phosphorylation before the convergence of signaling pathways which depend on the inducer; (ii) serine and threonine protein kinases, but not tyrosine protein kinases, are involved in distinct differentiation signaling pathways depending on the inducer; (iii) we have dealt with proteins which undergo changes

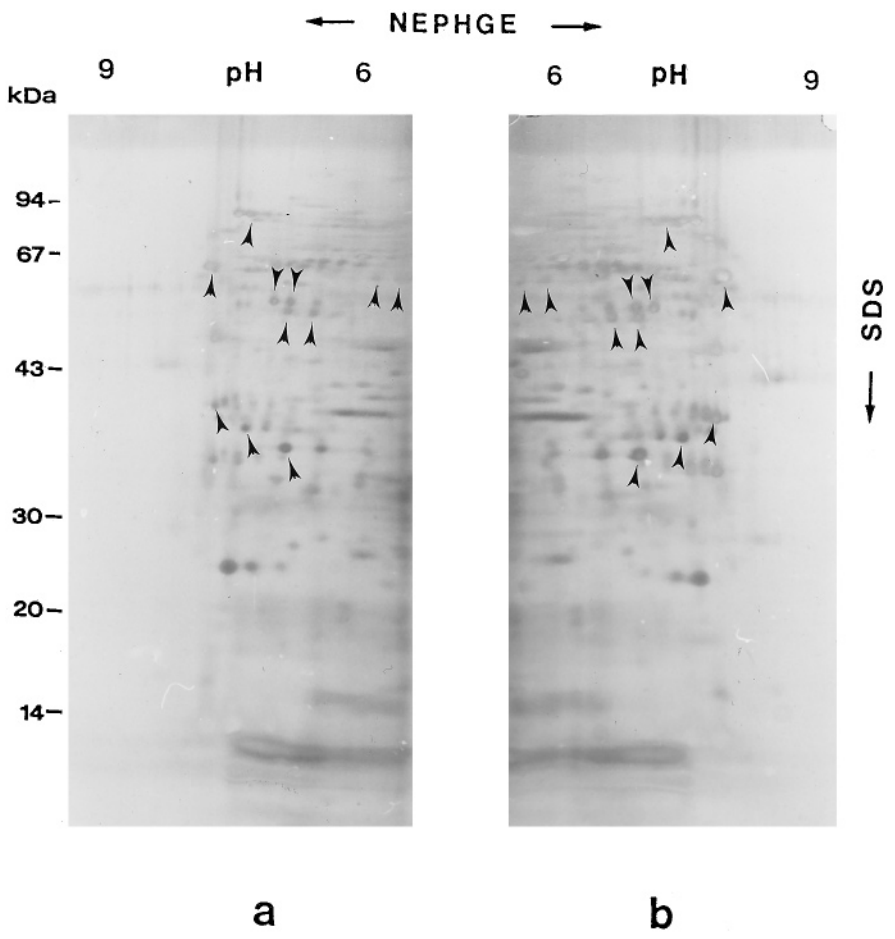

Figure 4. 2D electrophoresis of nuclear proteins isolated from control and differentiated HL-60 cells. The total nuclear proteins were isolated from (*a*) control and (*b*) differentiated HL-60 cells at 120 h after induction with 1.3% DMSO (v/v). The protein samples were prepared as described in 'Materials and methods'. The proteins were fractionated by 2D electrophoresis: NEPHGE (pH 6–9) in 5% PAG and SDS-PAGE in a 7.5–15% gradient polyacrylamide gel. The gel was stained using the nondiamine silver-staining method. The protein spots of abundant proteins that undergo tyrosine phosphorylation or dephosphorylation during differentiation are indicated by arrows.

in tyrosine phosphorylation after convergence of distinct differentiation signaling pathways, and these proteins are responsible for maintaining nuclear structures and their function in the differentiated phenotype. Nuclear proteins from eukaryotic cells can be resolved into about 1500 spots by 2D gel electrophoresis if they are labeled in vivo by ^{35}S-methionine [47]. This indicates that our 2D gel electrophoretic patterns, stained by CBB G-250, represent just the most abundant nuclear proteins which make up about 5% of the total number of nuclear proteins. In addition, the abundance of the analysed tyrosine-phosphorylated proteins suggests the possibility of detecting these proteins without using immunoprecipitation with antibodies against distinct tyrosine-phosphorylated proteins or with anti-phosphotyrosine antibodies. The abundance and drastic, apparently persistent changes in their tyrosine phosphorylation during HL-60 cell maturation into granulocytic phenotypes suggest that these proteins are not involved in signal receiving or induction mechanisms of differentiation, but most likely are structural proteins of the cell nucleus. It was shown earlier in cell extracts that during the maturation stage of murine monomyelocytic leukemia WEHI-3B cell differentiation that a striking increase in protein tyrosine phosphorylation is continuously counteracted by tyrosine dephosphorylation [48]. Our data concerning constitutive tyrosine phosphorylation in some of the abundant nuclear proteins of uninduced proliferating HL-60 cells are consistent with the presence of tyrosine-phosphory-

lated nuclear proteins in Abelson-transformed fibroblasts [35]. It should be noted that normal NIH 3T3 fibroblasts contain trace levels of tyrosine-phosphorylated nuclear proteins [35]. Long-term tyrosine-phosphorylated proteins of 75 and 53 kDa have been identified during HL-60 cell differentiation; however, they were not tested for nuclear localization [49]. The possibility that increased tyrosine phosphorylation or dephosphorylation during HL-60 cell maturation to granulocytes is a result of changes in the steady-state concentration of individual proteins seems to be unlikely for the following reasons: (i) SDS electrophoresis-scanning patterns of nuclear proteins isolated from control and differentiating HL-60 cells at 12, 24 and 96 h after induction are nearly identical (result not shown), except quantitative changes of some protein indicated in figure 1a; (ii) changes in tyrosine phosphorylation (fig. 1b,c) do not involve these proteins; (iii) 2D gel electrophoretic comparison of nuclear proteins from uninduced control and differentiated cells revealed that quantitative changes in the relative amounts of some proteins are very insignificant and do not involve proteins undergoing changes in tyrosine phosphorylation; (iv) in spite of some structural changes in the nucleus during HL-60 differentiation, the total nuclear protein content is stable [50]; (v) there are insignificant changes in the total protein synthesis of DMSO-treated HL-60 cells [51], whereas the activities of PTK and PTP 3–4 days after induction of differentiation increased threefold and sevenfold, respectively [39]; (vi) there are only 55 proteins out of 1438 (approximately 4%) during B-lymphocyte differentiation from pre-B stage to plasmocytes, the synthesis of which varied in intensity [47]. However, during the differentiation of HL-60 cells, 10 abundant nuclear proteins out of approximately 70 visible proteins in CBB G-250-stained gels are tyrosine-phosphorylated. Furthermore, the cessation of the cell cycle is an obligatory event in cell differentiation, the possibility that some tyrosine-phosphorylated or dephosphorylated nuclear proteins observed in this study are involved in the regulation of the cell cycle cannot be excluded. However, it has been shown that aclacinomycin A and marcellomycin, which inhibit HL-60 cell growth and induce differentiation, cause protein tyrosine phosphorylation, whereas adriamycin, which only inhibits HL-60 cell growth, does not [40].

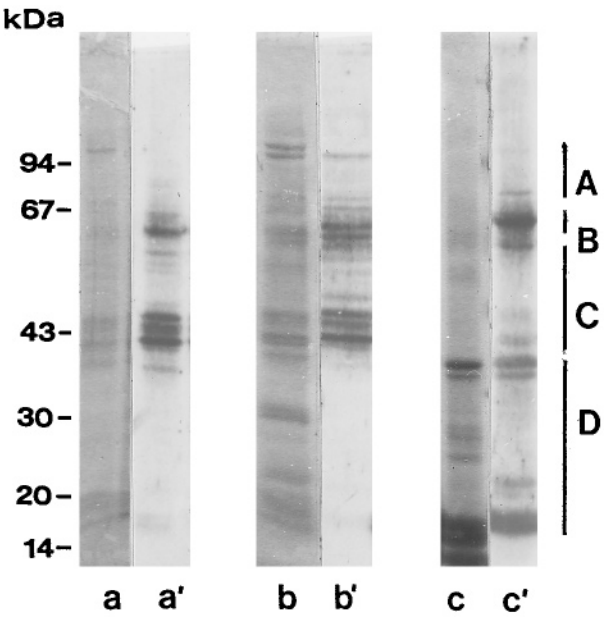

Figure 5. Tyrosine phosphorylation of nucleoplasm, nonhistone chromatin proteins and residual proteins of chromatin and nuclear matrix isolated from control HL-60 cells. Nuclei isolated from control HL-60 cells were sequentially extracted (a) with buffered 0.15 M NaCl (nucleoplasm proteins), and (b) with buffered 0.35 M NaCl (nonhistone chromatin proteins); (c) chromatin and nuclear matrix proteins obtained from salt-extracted nuclei as described in 'Materials and methods'. The proteins were resolved by SDS-PAGE in a 7.5–15% gradient polyacrylamide gel (a, b, c) and immunoblotted with anti-phosphotyrosine antibodies (a′, b′, c′).

Protein-DNA ratios generally range from 3 to 5, respectively, in the eukaryotic cell nucleus. The most abundant nuclear proteins are five histones (1:1 mass ratio to DNA) and some nonhistone chromatin proteins (mass ratio 1 to 2 per DNA), the number of which does not exceed 30 [52]. The total mass of the nucleoplasm and nuclear matrix proteins that is mainly represented by 20–30 abundant proteins consists of 30–50% of the nuclear proteins [46]. About 20 proteins of heterogeneous nuclear ribonucleoprotein (hnRNP) particles are localized in the nucleoplasm and partially within the chromatin [53]. Thus, the number of major proteins in the nucleoplasm, chromatin, nuclear matrix and envelope does not exceed 70–80. This number coincides with the number of proteins stained by Coomassie Brilliant Blue G-250. HL-60 nuclei were solubilized in 0.25% SDS-3% Nonidet P-40-8 M urea-50 mM DTT and the proteins resolved by 2D gel electrophoresis (fig. 3). The comparison of this 2D gel electrophoresis pattern to the 2D gel electrophoresis patterns of chromatin, nuclear matrix and hnRNP proteins obtained by others [46, 53] suggests that some of the abundant proteins in the 2D gel electrophoretic pattern (fig. 3) belong to the proteins from hnRNP particles. A high tyrosine phosphorylation signal is found in one spot with an approximate molecular mass of 110 kDa, seven spots in the molecular mass range of 60–70 kDa and three spots with a molecular mass of 35–40 kDa (fig. 3c,d) in Western blots, and their electrophoretic mobility coincides with that of the stained proteins (spots 3, 7, 8, 9, 12–15, 18–20 in fig.

3a,b). The molecular masses of all these proteins are higher than those of histones (14–21 kDa) or of the major nonhistone chromatin proteins (10–25 kDa), and the pI values of tyrosine-phosphorylated proteins with similar molecular masses such as lamins A, B and C (molecular masses 70, 68 and 60 kDa) are different. Furthermore, according to their extractability with 0.15–0.35 M NaCl, their abundance, molecular mass and pI values, some of the tyrosine-phosphorylated proteins might be hnRNP proteins.

Serine and threonine phosphorylation of histone H1 and core histones [54, 55], lamins A, C and B subtypes [56], nonhistone chromatin proteins [57] and hnRNP proteins [58] are well known, and these modifications are cell cycle-dependent. However, there is no information so far concerning tyrosine phosphorylation of structural proteins in cell nuclei. Recently, it has been shown that two RNA-binding proteins with a molecular mass of 68 kDa [59] and 62 kDa [60, 61] which bind Src by their SH2 domain are tyrosine-phosphorylated, and the association of p62 with RNA is regulated by this modification [61]. It is of interest that these two proteins share homology with the hnRNP protein K [59]. It has been shown that tyrosine phosphorylation of a 38-kDa A/B-type hnRNP protein selectively modulates its RNA binding [62]. It should be pointed out that three proteins undergoing changes in tyrosine phosphorylation during HL-60 differentiation (spots 18–20 in fig. 3c) are localized at the same position as A/B type hnRNP proteins in the 2D NEPHGE/SDS electrophoresis pattern obtained by others [53]. It has also been shown that members of the Src family of tyrosine kinases, that is Fgr, Fes, Fer and Lyn, localized in the nucleus are highly expressed in terminally differentiated neutrophils, monocytes and macrophages [15, 16, 19]. Moreover, the Nck SH2/SH3 adaptor protein is present in the nucleus and associates with the nuclear RNA-binding protein SAM68 [63]. More work is needed to specifically identify the nuclear proteins varying in tyrosine phosphorylation during differentiation. This study provides some clues which should be helpful in identifying the long-term tyrosine-phosphorylated nuclear proteins probably involved in the maturation of the granulocyte.

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