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Separation of Total Nuclear Proteins by an Improved Two-Dimensional Polyacrylamide Gel Electrophoresis —The Method and Applications to the Study of Induced Differentiation of HL-60 Cells—

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An improved experimental method was developed to solubilize total nuclear proteins and to separate them with reasonable resolution in two-dimensional polyacrylamide gel electrophoresis. The first dimension used urea-SDS-Triton X-100 system and the conventional Laemmli system was used for the second. The method was applied to the study of nuclear protein phosphorylation of human promyelocytic leukemia HL-60 cells upon short exposure to a differentiation inducer, 12-O-tetradecanoyphorbol 13-acetate. Three proteins of Mr 45 k, 56 k, and 60 k, designated as p 45, p 56, and p 60, respectively, exhibited increased phosphorylation on serine or threonine residues or both. A line of experimental evidence obtained supports the view that the phosphorylation of p 45 and p 56 may be directly involved in the early event in induction of differentiation of HL-60 cells.

KEY WORDS: Two-dimensional polyacrylamide gel electrophoresis/ Nuclear protein/ Phosphorylation; HL-60 cells/ Differentiation/ 12-O-tetradecanoylphorbol 13-acetate/

INTRODUCTION

The mechanism of external stimuli to induce specific responces of particular cells is the main interest in the current investigation of cell biology[6]. The stimuli are transmitted from the cell surface receptors to the nucleus where gene transcription and expression are modified. In this sequence of events, we are particularly interested in the induced modification of nuclear protein factors involved in the transcription reaction. Recently, much attention has been focused on the phosphorylation of nuclear proteins[7,8], and some experimental data now accumulating are on histones[9], nuclear matrix proteins[10], topoisomerase II[11], and lamin B[12]. However, a complete survey of changes of all nuclear proteins is hampered by the lack of experimental methods enabling solubilization and two-dimensional electrophoretic separation on total nuclear proteins. In the classical O'Farrell system[13], for example, aggregation of less soluble proteins is unavoidable in isoelectric focusing[14]. Modifications of the method were attempted for special classes of proteins such as ribosomal proteins, nuclear histones, and acid extracts of nuclear proteins[14], but a method applicable to total nuclear proteins in general is still unavailable due to aggregation of some protein fractions.

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This paper describes a modification of the two-dimensional polyacrylamide gel electrophoresis, where a urea-SDS-Triton X-100 system was used for the first dimension and the Laemmli system[15] for the second. The method was applied to the study of nuclear protein phosphorylation upon very short exposure of human promyelocytic leukemia HL-60 cells[16] to 12-O-tetradecanoylphorbol 13-acetate (TPA)[17], a differentiation inducer to macrophage[18,19,20]. The results obtained suggest that some nuclear proteins are phosphorylated as an early event in differentiation of HL-60 cells.

MATERIALS AND METHODS

Cells and Culture Conditions—HL-60 cells of passage 17 were provided by Dr. H. Henmi (Tohoku University, Sendai, Japan). The cells used here were passages 19 to 35 and had a doubling time of about 51 hr. The phagocytic cells after induction of differentiation with TPA were 79%. A high-passage HL-60 strain was obtained from Dr. K. Suzuki (Radiation Effects Research Foundation, Hiroshima, Japan), which has been continuously maintained for a long period of time and had a doubling time of about 25 hr. An HL-60 cell variant resistant to induced differentiation by TPA was isolated from the high-passage strain by limiting dilution and by further screeining non-differentiating cells after culturing for two and half months in the presence of 10^{-7} M TPA. The HL-60 subclone 22R exhibited only 2.6% differentiation after induction with TPA.

Cells were grown in plastic tissue culture flasks (Corning Plastics) in RPMI 1640 medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories), 2 mM glutamine, and 2% $NaHCO_3$. No antibiotics were used. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. In the usual maintaining, cells were seeded and passaged at cell densities of 2.5×10^5/ml and 2.5×10^6/ml, respectively. The cell cultures were periodically tested for mycoplasma by using mycoplasma stain kit (Flow Laboratories).

For the induction of differentiation, cells were incubated at a density of 8×10^5 cells/ml with, 1) TPA (Pharmacia) of a concentration of 10^{-8} M for two days; 2) retinoic acid (RA)(Sigma) of a concentration of 10^{-6} M for five days; 3) 1,25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) (a gift from Dr. M. Fukushima, Chugaiseiyaku, Tokyo) of a concentration of 10^{-7} M for two days.

For radioactive labelling, cells were washed once with phosphate-free RPMI 1640 medium (Flow Laboratories), incubated in the same medium at 37°C for 30 min, added with [^{32}P]-phosphate (0.1 mCi/ml)(Japan Radioisotope Association), and further incubated for 15 min. The incorporation of radioactivity into nuclear proteins was 10^{-2} cpm/cell after incubation for additional 60 min.

Assays of the Markers of Matured Cell Type—HL-60 cells are induced to differentiate into matured cells of the monocyte -macrophage lineage with TPA[18,19,20] or 1,25 $(OH)_2D_3$[21,22,23,24], and of the qranulocyte lineage with RA[25,26]. Phagocytosis and Nitro Blue Tetrazolium (NBT) reduction were used as the matured cell markers for these lineages, respectively.

HL-60 cells were incubated with an inducing reagent as described above in a dish

having been pretreated overnight with fetal bovine serum at room temperature. Cells were suspended by strong pipetting and collected in a microtube. The dish was repeatedly washed with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS (−)) for complete collection of the adherent cells. The whole collected cells were incubated at a cell density of 2×10^6/ml with a special type of polymethylmethacrylate microsphere[27] (a gift from Dr. S. Hosaka, Toray Industry Inc., Basic Research Laboratory, Kamakura, Japan) at a density of 5×10^7/ml for 12 hr. The cells were stained by the addition of 0.1 volumes of 0.2% fuchsine, and the phagocytic and viable cells were counted. Only the microspheres phagocytized into the cell remained unstained. The viable cells were at least 80%.

For NBT reduction studies 50 μl of cells suspended at 10^6 cells/ml of RPMI 1640 medium were mixed with an equal volume of 0.2% NBT (Sigma) dissolved in RPMI 1640 medium in the presence of 200 ng/ml TPA in a well prepared by sticking a silicone rubber sheet of 2 mm thick with a hole of 8 mm in diameter onto the regular slide glass. The mixture was incubated at 37°C for 20 min, fixed with methanol for 2 min, stained with 1% Safranin, and the percent of cells containing intracellular reduced blue-black formazan deposits was determined.

Preparation of the Nuclear Protein—For the isolation of cell nuclei, the sucrose-detergent procedure as described by Birnie[28] was employed except that 6 mM $CaCl_2$ was used in the buffer solution containing sucrose. Briefly, the washed pellet of cells were swollen in ice-cold hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4)) and homogenized in a glass Dounce homogenizer (Wheaton, 7 ml). The mixture was made 0.25 M in sucrose and centrifuged at $800\times g$ for 10 min at 4°C. The pellet was washed with Buffer A (6 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.4)) containing 0.25 M sucrose. The crude nuclear pellet so obtained was resuspended in 2.2 M sucrose, and centrifuged at $40{,}000\times g$ for one hr at 4°C in a swing rotor. The nuclear pellet was washed once again with 0.25 M sucrose in Buffer A in a microtube, and finally treated with 1% Triton X-100 to remove the outer nuclear membrane.

The total nuclear protein was extracted from the nuclear pellet according to LeStourgeon and Beyer[29]. Briefly, the purified nuclei were pelleted to the bottom of a 2.0-ml Potter type homogenizer, suspended in lysis buffer (2% SDS, 20 mM EDTA, 0.1% 2-mercaptoethanol, 20 mM Tris-HCl (pH 8.2)) gently with the Teflon pestle, and placed in boiling water for 3 min. After cooling to room temperature, the solubilized and reduced material was added with aqueous phenol, and the mixture was emulsified by homogenizing with the Teflon pestle at room temperature. After centrifugation, the phenol layer was dialyzed against a buffer solution (0.1% SDS, 0.25 M sucrose, 1% 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.4). Some protein precipitated during dialysis, but anyway the whole protein in the dialyzate was then intenionally precipitated by the addition of trichloroacetic acid (TCA) to 25%. After extraction of TCA with acetone, the precipitate was completely dissolved in 0.1 N NaOH on ice at 4°C, and the resultant solution was quickly neutralized by addition with shaking of 0.68 vol of a mixture to a final concentration of 3% SDS, 0.5% Triton X-100, pH 7. The complete solubilization of protein was accomplised by this procedure as verified by the absence of precipitate after extensive centrifugation. The proteinase inhibitor, phenylmethylsol-

fonyl-fluoride (PMSF) and the phosphodiesterase inhibitor, 3-isobutyl-l-methyl-xanthine (IMX) may be added here, but the data presented in this paper were obtained reproducibly without using them when the neutralized solution ws used quickly for electrophoresis. The sample for the two-dimensional electophoresis was prepared by further adding urea and dithiothreitol (DTT) to final concentrations of 5 M and 40 mM, respectively and incubated at 37°C for 30 min.

Two-Dimensional Polyacrylamide Gel Electrophoresis—The polyacrylamide gel electrophoresis in the first dimension used the urea-SDS-Triton X-100 system, and the Laemmli system[15] was used in the second[30].

The electrophoresis in the first dimension was performed in a rod gel with a diameter of 3.5 mm. The composition of the running buffer and those of the stacking and separation gels for the first dimension are given in Table 1. The electrophoresis at 125 V were for 5.5 hr and 7.2 hr, for the gels of concentrations of 5% and 9%, respectively. After electrophoresis the rod gel was removed from the glass tube and layered on the stacking gel of the second-dimension slab gel. A bent glass plate[31] was used to perform the reducing procedure of the rod gel on the stacking gel which had been treated in advance with 5% 2-mercaptoethanol in Laemmli stacking buffer of one half concentration[32]. After electrophoresis, the slab gel was stained with Coomassie Blue as usual. Artificially stained lines originated from scratches produced in removing the rod gel from the glass tube. We could avoid this by using an assembly constructed from longitudinally separated two halves of a tube with ground surfaces. In the analysis of ^{32}P-labelled protein samples, the gel was dried and a preflashed RX film

Table 1. Compositions for the urea-SDS-Triton X-100 system of polyacrylamide gel electrophoresis (first dimension)

Running buffer:	0.1% SDS, 1.44% glysine, 0.3% Tris-HCl (pH 8.3)	
Stacking gel:	4 M urea, 0.1% SDS, 0.3% Triton X-100 125 mM Tris-HCl (pH 6.8), 3% acrylamide	
Separation gel:	7 M urea, 0.1% SDS, 0.3% Triton X-100 100 mM Tris-HCl (pH 8.8), 5% acrylamide	(for high MW)
	125 mM Tris-HCl (pH 8.8), 9% acrylamide	(for low MW)

(Fuji Photo Film Co.) was exposed to it and developed by the conventional procedure.

Isolation and Acid-Urea Electrophoresis of High Mobility Group Protein—Isolated nuclei were homogenized in ice-cold 2% TCA and kept in ice for one hr to extract HMG proteins[33]. After insoluble material was removed by centrifugation, the supernate was added with TCA to a final concentration of 25% and stored in ice for one hr. The precipitate was washed twice with cold acetone, dissolved in 0.1 N NaOH, and the

resultant solution was made 2.5 M in urea and 5% in acetic acid. The sample so prepared was used for electrophoresis on acetic acid-urea slab gel containing 15% acrylamide, 2.5 M urea, and 5% acetic acid[34]. A pre-electrophoretic run was made at 130 V for 4 hr.

RESULTS AND DISCUSSION

Solubilization and Electrophoretic Separation of Total Nuclear Proteins—The method of LeStourgeon and Boyer[29] is excellent for extracting total nuclear proteins in a nondegraded form from isolated nuclei. The procedure takes advantage of the ability of aqueous phenol to specifically and rapidly concentrate protein in a phase separation from aqueous solution containing protein, nucleic acids, and polysaccharides. A complete recovery of proteins was assured with whole E. coli cells (data not shown). However, a small amount of proteins precipitated during the final dialysis step of removing phenol against a buffer solution containing 0.1% SDS, 1% 2-mercaptoethanol, 0.25 M sucrose, and 10 mM Tris-HCl (pH 7.4). Thus the whole protein including the precipitate was intentionally precipitated by the addition of TCA after the dialysis. This precipitate was then completely dissolved in a minimal volume of 0.1 N NaOH, and quickly neutralized by adding under shaking 0.68 vol of a solution which contained 7.4% SDS and 1.2% Triton X-100. The simultaneous addition of SDS and Triton X-100 in neutralization was effective in preventing the onset of aggregation. The solution was then quickly used to prepare the sample for electrophoresis. No protein aggregates were trapped on gel top during electrophoresis.

High Resolution of the Modified Two-Dimensional Polyacrylamide Gel Electrophoresis—Our aim here is to develop an improved experimental procedure of two-dimensional gel electrophoresis, which achieves a reasonable resolution without onset of aggregation of any nuclear proteins. For the second dimension, choices other than the Laemmli SDS system are scarcely possible due to the requirement of measuring the molecular size. Thus, the consideration to fulfil the aim is focused on the first dimension: 1) be based on a principle different from that of the Laemmli system for good resolution; 2) free from protein aggregation. Off-diagonal alignment of protein spots in the final result gives a criterion of good resolution. Apparently we cannot use isoelectric focusing[13] for total nuclear proteins due to inevitable aggregation of some proteins. One possibility is to use urea and non-ionic detergent such as Triton X-100 in addition to SDS to increase the solubility on one hand and hopefully to differentiate the electrophoretic characteristics from the SDS system, on the other hand. Such a combination of reagents was actually used in the analysis of nuclear histones[14]. Further important improvements were made as shown below by the finding that the concentration of Tris buffer very much affects the resolving power.

Figure 1 shows the results on total nuclear proteins of HL-60 cells. If we use the concentration of 375 mM for Tris buffer for the separation gel in the first dimension as the conventional Laemmli system, the resolution was poor (Fig. 1(A)). However, much improvement can be seen when the buffer concentration was decreased to 100 mM (Fig. 1(B)) as indicated by many off-diagonal spots. The optimum buffer concentration

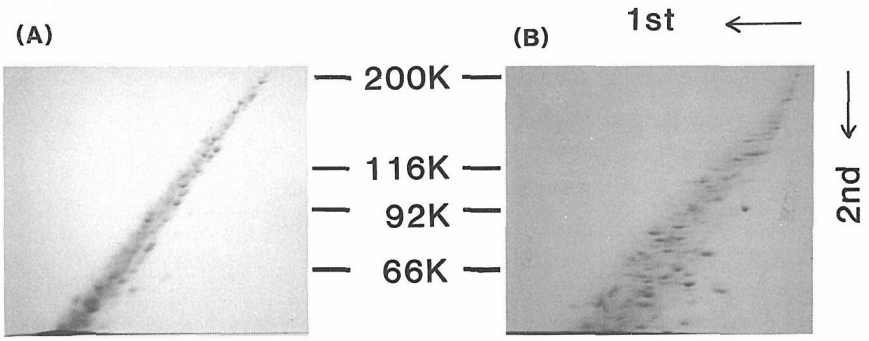

Figure 1. The Coomassie Blue stainings of the modified two-dimensional polyacrylamide gel electrophoresis on the total nuclear proteins of human promyelocytic leukemia HL-60 cells. Panel (A) shows the result when a concentration of 375 mM Tris-HCl (pH 8.8) was used in the separation gel (5% acrylamide) of the urea-SDS-Triton X-100 system for the first dimension, and panel (B) shows the improvement in the resolution when a lower concentration of Tris-HCl buffer (100 mM) was used. The numbers indicate the marker molecular weight.

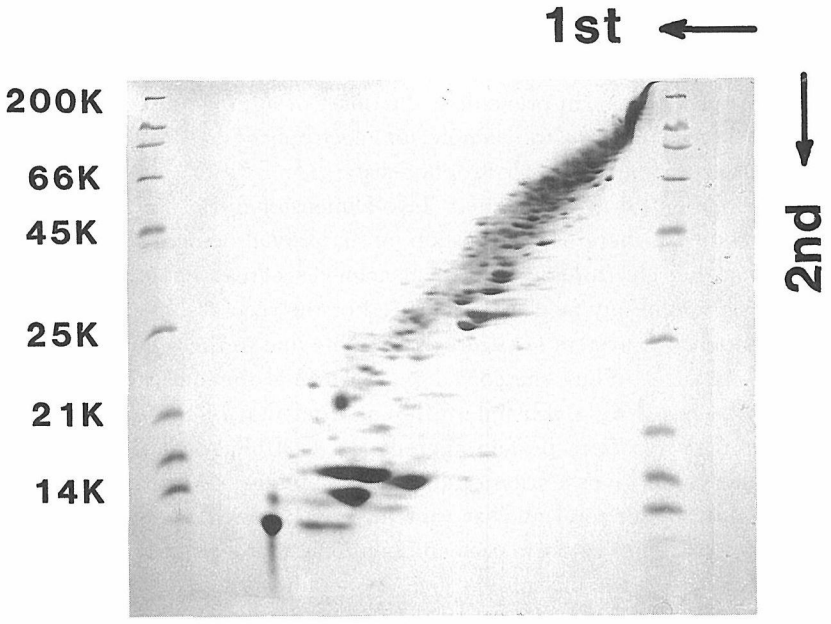

Figure 2. Analysis of the nuclear proteins in the low molecular weight region of HL-60 cells by the modified two-dimensional polyacrylamide gel electrophoresis. A concentration of 125 mM Tris-HCl (pH 8.8) was used in the gel (9% acrylamide) for the first dimension. The numbers indicate the marker molecular weight.

depended on the gel concentration. For the protein analysis in the high molecular weight region ($40–200\times10^3$), a 5% polyacrylamide gel was used with 100 mM Tris-HCl, while a 9% gel was used with 125 mM Tris-HCl in the low molecular weight region ($15–60\times10^3$)(Fig. 2). This result indicates that the binding of SDS in the presence of urea

and Triton X-100 depends on each protein characteristics other than the molecular weight if the concentration of Tris buffer is regulated appropriately. We first expected a better resolution if unreduced protein samples are used in the first dimension, because the specific mode of intramolecular disulfide bonding may affect the conformation and hence binding of the ionic detergent SDS. However, the results were always smeared due perhaps to the frequent exchange of disulfide bonds or the variability in the amount of bound SDS or both.

Application of the Modified Two-Dimensional Polycrylamide Gel Electrophoresis to the Analysis of Nuclear Protein Phosphorylation upon Induction of Differentiation of HL-60 Cells—Human promyelocytic leukemia HL-60 cells[16] are induced to differentiate into monocyte-macrophage, granulocyte, and eosinophil. The typical inducers are 1,25 $(OH)_2D_3$[22,23,24] and TPA[18,20] for monocyte-macrophage, RA for granulocyte[25,26], and slightly alkaline growth medium (HEPES buffer, pH 7.8) for eosinophil[35]. The molecular mechainsms involved in the stimulation of specific gene expression by the external differentiation signals stil remain to be clarified (see ref. 36 for recent reviews). Phosphorylation of nuclear proteins is considered a step of external signal transduction[8], but a general survey of total nuclear modifications has been hampered by the lack of appropriate experimental method of two-dimensional electrophoresis. The method developed in the present study may open a way for such an approach.

Figure 3 shows the results of Coomassie Blue staining as a function of duration of the TPA treatment. Some spots exhibited significant increase in the amount (Mr 93 k, 140 k, and 160 k), and a spot showed quick decrease and eventually disappeared after 24 hr.

Figure 4 shows the autoradiograms of the two-dimensional electrophoresis of HL-60 nuclear proteins demonstrating an enhanced phosphorylation upon incubation in 10 nM TPA for a short period of 60 min. The three spots indicated by the arrows are

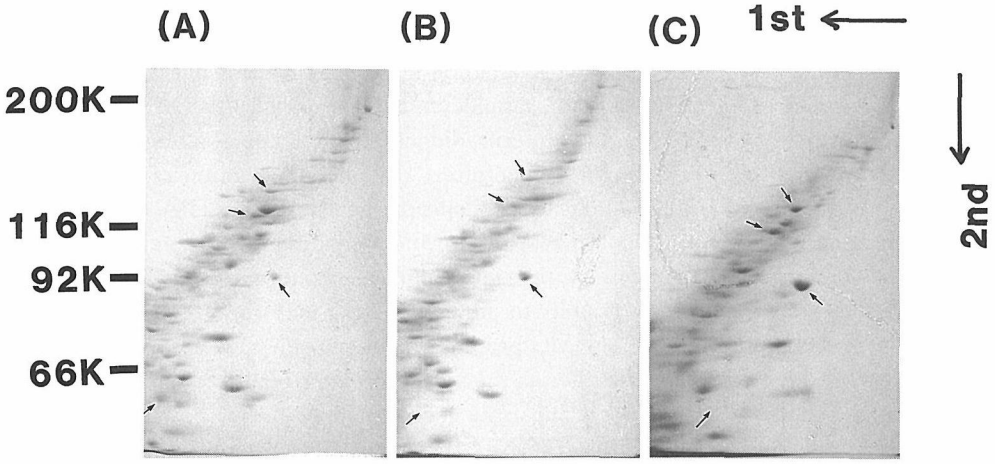

Figure 3. Coomassie Blue stainings of the two-dimensional polyacrylamide gel electrophoresis on the nuclear proteins of HL-60 cells treated with 10^{-8} M TPA for (A) 0 time, (B) 30 min, and (C) 24 hr. The numbers indicate the marker molecular weight. The arrows indicate representative spots of increased density (Mr, 93 k, 140 k, and 160 k) and decreased density (Mr, 62 k).

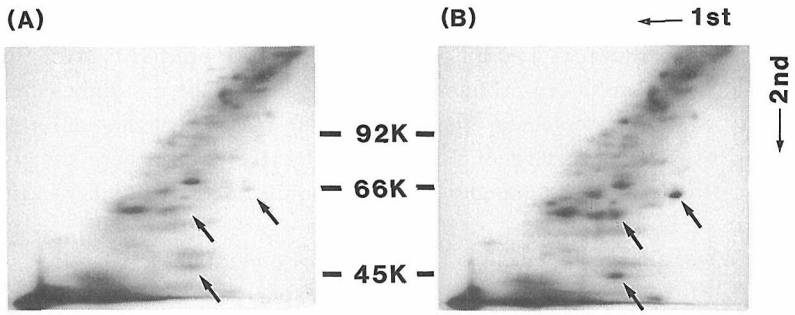

Figure 4. Autoradiograms of the two-dimensional polyacrylamide gel electrophoresis on the nuclear proteins of HL-60 cells treated with 10^{-8} M TPA for (A) 0 time, (B) 60 min, in the presence of [^{32}P]-phosphate. The arrows indicate the representative spots of increased density. Their molecular weights are 45 k, 56 k, and 60 k. The numbers indicate the marker moleculare weight.

those exhibiting the most significant increase in phosphorylation whose molecular masses are 45, 56, and 60 k Da. The proteins are designated as p 45, p 56, and p 60, respectively. We also investigated the proteins in the low molecular weight region ($15–60\times10^3$) but changes in phosphorylation by the TPA treatment were not detected (data not shown). The radioactivity of p 60 was 60% higher than the control. In contrast, the differentiation-resistant clone 22R showed an increase of only 20%. The background radiactivity was not considered in the estimate. The phagocytic cells after differentiation induction were 12% for 22R in contrast to 80% for the HL-60 cells of low passages. The spots of increased phosphorylations all disappeared by an alkali treatment of the gel at 55°C for 2 hr[37], indicating that the phosphoamino acids were serine or threonine or both, but not tyrosine.

Phosphorylation of nuclear proteins was also investigated on HL-60 cells incubated with RA or 1,25 $(OH)_2D_3$. Incubation in 10^{-7} M 1,25 $(OH)_2D_3$ for two days converted HL-60 cells about 70% phagocytic, and incubation in 10^{-6} M RA for five days induced 75% of HL-60 cells to differentiate into granulocyte as measured by the NBT reduction activity. However, phosphorylation patterns did not exhibit appreciable changes by the two treatments (data not shown). Therefore, the phosphorylation of the nuclear proteins, p 45, p 56, and p 60, is likely to be a specific event for the TPA treatment.

Much attention has been focused recently on the activation of protein kinase C by TPA by the similar mechanism as the intracellular second messenger DAG[38,39]. However, OAG by itself was unable to induce HL-60 maturation[40,41,42]. In this connection, it was reported that the HMG protein 17 was phosphorylated by kinase C in a single seryl residue equally well either as the pure protein or bound to nucleosomes[43]. Calf thymus histone is also a good substrate for protein kinase C[44]. Interestingly, amiloride, a frequently used inhibitor of Na^+/H^+ exchange, rapidly inhibited TPA-induced transferrin receptor phosphorylation *in vivo* and TPA-induced adhesion of HL-60 cells[45], another marker of differentiation.

By the treatment of HL-60 cells with OAG for 60 min at a concentration of 40 μg/ml, a condition that that protein kinase C is activated in intact HL-60 cells[43], the

three nuclear proteins referred to above were not phosphorylated. This result indicates that the proteins may not be the direct substrate for protein kinase C. Phosphorylation of nuclear proteins on serine and threonine residues may be possible by protein kinase M, a soluble low molecular weight form of protein kinase C[46] as suggested by P. Hornbeck et al.[12] Another evidence supporting the view was obtained on HMG proteins extracted with 2% TCA and analyzed by acid-urea gel electrophoresis. The proteins were not phosphorylated by the TPA treatment. Histone H1 was not phosphorylated either (data not shown). Finally we investigated the effects of amiloride on the phosphorylation of p 45, p 56, and p 60. Figure 5 shows the results when 10^{-3} M amiloride was added during ^{32}P-labelling and TPA incubation. The induced differentiation as measured by adhesion was 80% inhibited in this condition[45]. It is clearly seen from Figure 5 that phosphorylation with 10 nM TPA on p 45 and p 56 was completely inhibited, while that of p 60 was little affected.

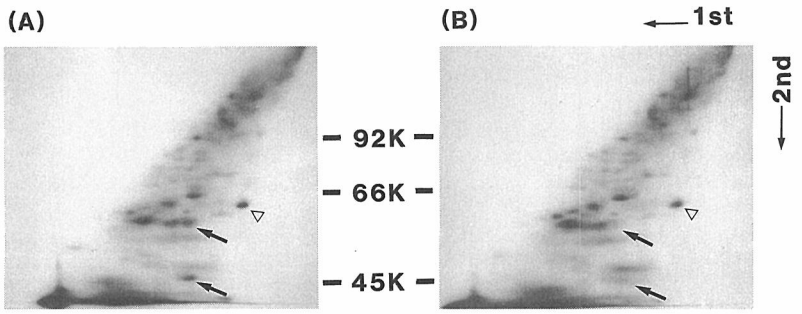

Figure 5. Phosphorylations of nuclear proteins, p 45 and p 56, of HL-60 cells by incubation with 10^{-8} M TPA for 60 min (panel (A)) were inhibited by the addition of 1 mM amiloride (panel (B)) as indicated by arrows. The phosphorylation of p 60 was unaffected as pointed out by the triangle. The numbers indicate the marker molecular weight.

Summing up, we analyzed the effects of short term incubation of HL-60 cells with differentiation inducers, TPA, $1,25(OH)_2D_3$, and RA on the nuclear proteins by using the newly modified two-dimensional gel electrophoresis. Three proteins of Mr 45 k, 56 k, and 60 k exhibited increased phosphorylation, which was sepcific to the TPA treatment, much less for TPA-resistant HL-60 derivative 22R, and not acieved by the OAG treatment. The phosphorylated amino acid residues were serine or threonine or both, but not tyrosine. The phosphorylated proteins were neither HMG proteins nor histones. Particularly, the phosphorylation of p 45 and p 56, but not p 60, were inhibited by amiloride. These results are consistent with the notion that the phosphorylation of nuclear proteins p 45 and p 56 may be related to the early event in induction of differentiation of HL-60 cells with TPA. Apparently further investigations are necessary to identify the proteins and to elucidate their bichemical functions in nuclei.

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(17) Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; RA, retinoic acid, $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; NBT, Nitro Blue Tetrazolium; PBS (−), Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline; TCA, trichloroacetic acid; DTT, dithiothreitol; HMG, high mobility group; DAG, diacylglycerol; OAG, 1-oleoyl-2-acetylglycerol.
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