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Specific Protein Phosphorylation in Human Promyelocytic HL-60 Leukemia Cells Susceptible or Resistant to Induction of Cell Differentiation by Phorbol-12-myristate-13-acetate[1]

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ABSTRACT

The pattern of protein phosphorylation induced by phorbol-12-myristate-13-acetate (PMA) was analyzed by two-dimensional gel electrophoresis in human HL-60 leukemia cells, which are susceptible to induction of cell differentiation by PMA, and in cells from an HL-60 cell variant designated R-94 that are resistant to such an induction. Protein phosphorylation was detected by observing either a rapid acid-directed charge shift of [^{35}S]methionine-labeled protein or an increase in the amount of phosphate label in a ^{32}P-labeled protein. The results indicated that PMA at 10^{-7} M causes within 30 min after treatment the phosphorylation of at least ten different proteins in both the HL-60 and R-94 cells. Among these ten phosphorylated proteins, we identified a major cytoplasmic polypeptide (M_r ~64,000), a cytoskeletal protein (M_r ~56,000), a nonmuscle myosin light chain, and two proteins (M_r ~60,000 and 64,000) localized in or around the cell nucleus. Phosphoamino acid analysis of six of the ten phosphoproteins showed that they contain phosphoserine. None of these proteins contained phosphotyrosine or phosphothreonine.

The R-94 cell variant was found to be capable of increased protein phosphorylation after PMA treatment; however, the level of phosphate incorporation reached only the level of the untreated HL-60 cells and thus fell far short of the level observed in the HL-60 cells after PMA treatment. It is suggested that the basis for the acquired resistance in R-94 cells towards induction of cell differentiation by PMA is a block in signal transmission involving phosphorylation of nuclear protein(s) following the binding of the inducer PMA to its receptor (protein kinase C).

INTRODUCTION

Tumor-promoting phorbol diesters and related agents can in some human cells elicit an inhibition of cell replication and an induction of cell differentiation (1–11). In the promyelocytic HL-60 leukemia cells, which are frequently used in cell differentiation studies (12), these chemicals cause the cells to acquire a mature phenotype that resembles that of monocytes and macrophages (4, 13–15).

The biological activity of the phorbol diesters is initiated following their binding to high affinity and saturable receptors (16–19). This binding in turn activates a calcium- and phospholipid-dependent protein kinase, termed protein kinase C (20–22). Recent studies have demonstrated that both the phorbol diester receptor and protein kinase C reside in a single protein (22–24). It is thus possible that protein phosphorylation induced by these agents is the key element that initiates the differentiation processes in the HL-60 and perhaps other susceptible cells. In this context, we note that, in the HL-60 cells, PMA,[3] a phorbol diester prototype, causes the phosphorylation of a number of proteins within a short time after treatment (25–27).

The current studies were undertaken to identify phosphorylated proteins that are associated with the induction of cell differentiation in the HL-60 cells. Using two-dimensional gel electrophoresis, we compared the patterns of the PMA-induced phosphorylated proteins in strains of HL-60 cells that are either susceptible or resistant to induction of cell differentiation by phorbol diesters. The HL-60 cells were incubated with PMA for only 30 min to allow the detection of protein phosphorylation associated with the induction rather than with the expression of cell differentiation.

MATERIALS AND METHODS

Cells and Culture Conditions. HL-60 cells were provided by Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD) (12). An HL-60 cell variant resistant to induction of cell differentiation by phorbol diesters (14, 15, 28, 29), designated R-94, was isolated by culturing HL-60 cells for 94 subcultures (at 5- to 8-day intervals) in the presence of increasing concentrations of PMA (Chemicals for Cancer Research, Eden Prairie, MN). The R-94 cells were subcultured 4 to 6 times in the absence of PMA prior to their use in the various experiments. Cells for the differentiation studies were incubated at 2×10^6 per 100-mm bacterial or tissue culture Petri dishes (Falcon Plastics, Oxnard, CA) in 10 ml of RPMI 1640 supplemented with 20% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, NY) (GIBCO) at 37°C in an atmosphere of 5% CO_2 in air in humidified incubators. A day after seeding the cells, the cultures were treated with either 10^{-9} M or 10^{-7} M PMA in a final concentration of 0.01% (v/v) dimethyl sulfoxide in culture medium. This concentration of DMSO did not affect cell growth, differentiation, or the expression of the various proteins.

Labeling of Cells and Two-Dimensional Gel Electrophoresis. Cells for two-dimensional gel electrophoresis were incubated in flat-bottomed multiwell plates at 10^6 cells per well in RPMI 1640 medium (GIBCO selectamine kit) supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics, but without methionine or phosphate where appropriate. After the cells were seeded, they were labeled with either [^{35}S]methionine (60 μCi/ml) or [^{32}P]phosphate (0.5 mCi/ml) (New England Nuclear) for 4 h. PMA at 10^{-9} M and 10^{-7} M was added for 30 min to the labeled cells.

At the end of the PMA treatment, the cells were harvested and solubilized in 60 μl of a solution containing 9 M urea, 4% Nonidet P-40, 2% mercaptoethanol, and 2% ampholytes (pH 9 to 11; LKB Bromma, Sweden) at a final pH of 9.5. Two-dimensional electrophoresis was

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[3] The abbreviation used is: PMA, phorbol-12-myristate-13-acetate.

performed with the 7- x 7-in ISO-Dalt system (30, 31), with a 1:1 mixture of wide-range Pharmalyte (Pharmacia Fine Chemicals, Piscataway, NJ) and Servalyte (Serva Feinbiochemica, Heidelberg) used as ampholyte. The gels were then dried and autoradiographed on Kodak XAR-2 film.

Extraction of Proteins and Phosphoamino Acid Analysis. Portions of the dried gels containing phosphoproteins were excised with a 0.25-in cork borer. The gel pieces were rehydrated in 1 ml of 0.05 M NH_4HCO_3:0.1% sodium dodecyl sulfate. Proteins were eluted essentially as previously described by Beemon and Hunter (32) except that the gel pieces were not homogenized. The final trichloroacetic acid pellet was resuspended in 0.5 ml of 6 N HCl by heating to 100°C for 1 min. Proteins were hydrolyzed at 110°C for 1 h under N_2, and the phosphoamino acids were purified as described by Cooper and Hunter (33). Purified phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates (MCB-EM Science) at pH 3.5 in pyridine:acetic acid:H_2O (5:50:945) for 50 min at 1 kV. The positions of unlabeled phosphoamino acid standards were determined by staining with ninhydrin. ^{32}P-labeled phosphoamino acids were visualized by fluorography with Dupont Cronex lightening plus intensifying screens. The plates were aligned with the final autoradiographs by using markings made with phosphorescent ink.

Evaluation of Morphological Differentiation. For morphological evaluation of cell differentiation, control and treated cells were removed from the Petri dishes, pelleted by centrifugation, and resuspended in 1 ml of fresh medium. Approximately 0.1 ml of the cell suspension was spread on a glass microscope slide, air dried, and stained with Wright-Giemsa. Differential cell counting was performed on 200 to 400 stained cells from at least two preparations for each experimental point.

Lysozyme and Nonspecific Esterase Activities. Lysozyme activity was determined by a decrease in turbidity of a cell suspension of *Micrococcus lysodeikticus*, measured at 450 nm at 25°C, as described (34). The reaction mixture (3 ml) contained 2.5 ml of substrate (0.12 mg of bacteria per ml of 67 mM potassium phosphate buffer, pH 6.24) and 0.5 ml of cell-free supernatant from control or treated cultures. The μg equivalent of lysozyme activity was estimated by comparing the cell-free supernatant activity with that of a standard activity curve with purified hen egg white lysozyme used as a standard (Sigma Chemical Co. Bulletin L-6876). Results are expressed in μg equivalents/10^7 cells/10 ml of medium.

Nonspecific esterase activity was determined cytochemically with α-naphthyl acetate used as the substrate (35). Cells spread on a microscope slide were fixed with cold formalin:acetone and incubated in a Coplin jar for 45 min at room temperature in a staining solution that contained 63.5 ml of phosphate buffer (0.1 M, pH 7.6), 4.2 ml of hexazotized ρ-rosalinin (obtained by mixing an equal volume of 4% sodium nitrite and 4% ρ-rosalinin), and 3.5 ml of α-naphthyl acetate solution (28.6 mg/ml of ethylene glycol monomethyl ether). Prior to use, the pH of the staining solution was adjusted to pH 6.1, and the solution was filtered through filter paper to remove precipitated material.

Reactivity with OKM1 Monoclonal Antibody. The murine OKM1 monoclonal antibody, which exhibits reactivity with human blood monocytes and granulocytes (36), was obtained from Ortho Pharmaceutical Corp., Raritan, NJ. Reactivity with OKM1 antibody was detected in the presence of a fluorescein-labeled goat anti-mouse antibody. The immunofluorescence was determined after 10^6 cells, suspended in 200 μl of cold phosphate-buffered saline, were incubated with 5 μl of reconstituted OKM1 monoclonal antibody (diluted 1:40 with buffer) for 30 min at 4°C. The cells were washed twice with ice-cold buffer and resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin for 30 min at 4°C. After 2 additional washes with buffer containing 30% glycerol, cell suspensions of 0.05 ml were dropped on glass slides, and the cells were covered with a coverslip and examined for reactivity with the antibody. Each experimental point was based on an analysis of 200 cells from two separate preparations.

RESULTS

Susceptibility of HL-60 and R-94 Cells to Induction of Cell Differentiation by PMA. Six-day incubation of HL-60 cells in the absence of PMA and R-94 cells in the absence or presence of 10^{-9} M PMA yielded a similar cell density, and the cultures contained a low percentage of cells that (*a*) attached to the surface of tissue culture Petri dishes (<5%), (*b*) exhibited morphological cell maturation (6 to 8%), (*c*) reacted with the anti-cell-surface OKM1 monoclonal antibody (<4%), and (*d*) stained for nonspecific esterase (2 to 3%). Cell-free supernatants of these cultures also exhibited a low lysozyme activity of 2 to 3 μg equivalents/10^7 cells/10 ml of medium (Table 1). On the other hand, HL-60 cells treated with 10^{-9} M PMA for the same time period yielded a reduced cell density, and the treated cells exhibited a phenotype that resembles that of monocytes and macrophages. This phenotype was characterized by the ability of the cells to (*a*) attach to the surface of the tissue culture Petri dish (>80%), (*b*) express morphological cell maturation (82%), (*c*) react with the anti-cell-surface OKM1 monoclonal antibody (73%), (*d*) stain for nonspecific esterase activity (75%), and (*e*) exhibit an increased lysozyme activity that was more than 30-fold higher than the corresponding control.

Incubation of the HL-60 cells for 6 days with 10^{-7} M PMA, rather than with 10^{-9} M PMA, resulted in an excessive cell death and in the acquisition of a mature phenotype in the surviving cells. These same conditions barely affected the replication of the R-94 cells. After the 6 days of treatment with 10^{-7} M PMA, only a small number of the cells (<10%) reacted with the differentiation-specific OKM1 monoclonal antibody, and there was less than 3-fold increase in lysozyme activity.

Protein Phosphorylation in Control or PMA-treated HL-60 Cells. The pattern of protein synthesis in the HL-60 or R-94 cells was observed by two-dimensional electrophoresis of [^{35}S]methionine-labeled proteins. Protein phosphorylation in these gels was detected by either observing a rapid acid-directed charge shift of [^{35}S]methionine-labeled protein or by an increase in the amount of phosphate label in a ^{32}P-labeled protein. In each case, the two indications are consistent. The analyzed proteins do not represent the entire polypeptide population of the cells, since only approximately 10% of the cellular polypeptides were examined under the conditions used for two-dimensional electrophoresis.

Treatment of HL-60 of R-94 cells with 10^{-7} M PMA for 30 min did not affect the synthesis or phosphorylation of the majority of their proteins. Rapid phosphorylation was, however, detected in

Table 1

Expression of cell differentiation markers in HL-60 and R-94 cells after 6 days of treatment with 10^{-9} M PMA

Parameters	HL-60 cells		R-94 cells	
	Control	PMA treated	Control	PMA treated
Cell no. (× 10^5/ml)	21	0.5	24	23
Cell attachment (%)[a]	<5	>80	<5	<5
Morphological mature cells (%)	7	82	6	8
Cells reacting with OKM1 monoclonal antibody (%)	<4	73	<4	<4
Cells stained for nonspecific esterase activity (%)	3	75	2	3
Lysozyme activity (μg equivalents/10^7 cells/10 ml)	3	110	2	2

[a] Cell attachment to the surface of the tissue culture Petri dish.

at least ten proteins in the PMA-treated HL-60 cells (proteins labeled 1 to 3, 6, and 9 to 14 in Figs. 1 and 2). These proteins range in molecular weight from 12,000 to 120,000 (Fig. 1) and represent a wide cross-section of cellular proteins. Of these, protein P:1 (M_r ~64,000) is a cytoplasmic polymorphic protein characteristic of normal and leukemic human WBC but not of other types of cells that are derived from muscle, brain, or connective tissue.[4] P:2 (M_r ~56,000) is a major cytoskeletal protein of human leukocytes, which in HL-60 cells appeared to gain several phosphates upon PMA treatment (37). In contrast to the above tissue-specific proteins, P:3 (M_r ~60,000) is found to be expressed in virtually all cultured human cells and consists of a double set of spots separated in P_i by approximately three charge units; both forms shift in the acid direction after PMA treatment and show increased phosphate labeling. Cytochalasin B enucleation experiments indicated that P:3 is at least partially localized in or around the nucleus. The same experiments indicate that P:9 (M_r ~64,000) is almost entirely nuclear (37). Another protein, P:14 (M_r ~17,000) is a complex of spots that contain one of the two common types of nonmuscle myosin light chains. Each of the two forms (spots marked *14* and a similar set immediately below to the *right* in Figs. 1 and 2) was present in single and double phosphorylated forms, the latter of which was significantly increased after PMA treatment. P:6 (M_r ~15,000) is a major low-molecular-weight phosphoprotein and is probably the protein referred to by Feuerstein and Cooper (25) as the protein whose phosphorylation is a major effect of PMA treatment in HL-60 cells. Unlike the above proteins, P:10 to 13 are as yet uncharacterized proteins. Two additional proteins P:5 (M_r ~35,000) and P:8 (M_r ~90,000) are examples of two phosphorylated proteins that were not affected by treatment of the HL-60 PMA. Proteins P:4, 5, and 7 are more easily detected by ^{32}P than ^{32}S label, indicating that they are either minor constituents or poor in methionine.

HL-60 cells treated with 10^{-9} M PMA, a dose two orders of magnitude lower than in the previous experiments, did not result in a detectable increase in protein phosphorylation. Studies in which HL-60 cells were exposed to 10^{-7} M and 10^{-9} M PMA in culture conditions identical to those used in the differentiation studies confirmed that the methionine-free medium and the culture wells used in the labeling studies were not responsible for the undetected increase in protein phosphorylation at the lower PMA dose (data not presented). Thus, to observe an increase in protein phosphorylation with the lower PMA dose requires either longer treatment with PMA or our experimental conditions were not sensitive enough to allow such a detection. The latter is more likely, since at 10^{-7} M, the levels of phosphorylation do not appear to increase (and, in general, decline) after 30-min incubation.[4]

Protein Phosphorylation in Control or PMA-treated R-94 Cells. In the R-94 cells that are resistant to induction of cell differentiation by PMA (Table 1), the total level of protein phosphorylation was not found to be lower than in the parental HL-60 cells, indicating that there is not a general defect in protein phosphorylation. The control R-94 cells usually exhibited a reduced level of phosphorylation of the ten proteins that are altered in HL-60 cells (Fig. 1). P:1 and P:6, the two most responsive proteins in terms of phosphorylation in the parental HL-60 cells, were almost unphosphorylated. Furthermore, treatment of R-94 cells with 10^{-7} M PMA resulted in some increase in phosphorylation levels, but these only reached the levels of control HL-60 cells (Fig. 2). In the case of P:2 and P:12, the proteins were synthesized at much reduced levels in both the control and treated variant cells; thus detecting changes in the phosphorylation of these proteins was difficult. The capacity to respond to PMA by increasing phosphorylation of specific proteins may therefore be intact, although the final levels attained are much lower due to the existence of lower (almost undetectable) starting PO_4 levels for this protein set. A number of other changes in the pattern of gene expression are also observed in R-94 compared to HL-60 cells; particularly prominent is a novel unphosphorylated group of M_r 40,000 acidic spots (marked as *ovals* in Fig. 2), indicating that perhaps a series of events rather than one mutation is responsible for the variant's altered behavior with respect to PMA.

Phosphoamino Acid Analysis of Phosphorylated Proteins from Control and PMA-treated HL-60 Cells. Phosphoamino acid analysis was performed on eight phosphoproteins (P:1 to 8) excised from gels prepared from either control or PMA-treated HL-60 cells (Fig. 1). Seven of these proteins obtained from the control (labeled as C1, C2, C3, C4, C6, C7, and C8) and the corresponding seven from the PMA-treated cells (T1, T2, T3, T4, T6, T7, and T8) were found to be phosphorylated at a serine residue. None of these proteins contained detectable amounts of phosphothreonine or phosphotyrosine (Fig. 3). C8 and T8, which correspond to P:8 in Fig. 1, represent a protein that is not affected by PMA treatment. Another protein, P:5 (labeled in Fig. 3 as T5), which is also not affected by PMA, contained similar amounts of phosphoserine and phosphotyrosine. P:5 is a nuclear phosphoprotein that often streaks in the focusing dimension due to poor solubility.

DISCUSSION

Cell variants derived from differentiation-susceptible cells, which do not respond specifically to these inducers, are useful for studies of the mechanism whereby phorbol diesters and related tumor promoters induce differentiation. We have isolated such a cell variant designated R-94 from the HL-60 cell line (14, 15, 17, 28, 29). These cells, like the HL-60 cells, are susceptible to induction of differentiation by dimethyl sulfoxide (12, 14) but, unlike the parental cells, these R-94 cells are resistant to induction of differentiation by PMA and teleocidin (14, 15), a nonphorbol ester tumor promoter (38).

The biological activity of the phorbol diesters in these as well as in other susceptible cells is initiated following their binding to high-affinity and saturable receptors (15–19). This binding, in turn, activates a calcium- and phospholipid-dependent protein kinase, termed protein kinase C (20–22). Recent studies have demonstrated that both the phorbol diester receptor and protein kinase C reside in a single protein (22–24). Differences in protein phosphorylation patterns in HL-60 and R-94 cells after PMA treatment may thus clarify the role of specific protein phosphorylation in the control of cell differentiation in the HL-60 and other related cell types.

A two-dimensional gel electrophoretic analysis of the phosphorylation patterns of the HL-60 and R-94 cell proteins indicated that, in both cell types, PMA causes within a short time (30 min)

[4] N. L. Anderson, M. A. Gemmell, P. M. Coussens, S.-i. Murao, and E. Huberman, unpublished results.

an increased phosphorylation of at least ten different proteins. Among these we identified a major cytoplasmic polypeptide (P:1), a cytoskeletal protein (P:2), myosin light chains (P:14), and proteins apparently associated with the cell nucleus (P:3 and P:9). In contrast to results from studies with some other types of cells, none of a series of six tested proteins derived from either control or PMA-treated HL-60 cells contains phosphotyrosine, which is associated with the activity of either the growth factor-controlled or oncogene-type protein kinases (39–41).

The R-94 cells were capable of exhibiting an increased protein phosphorylation after PMA treatment, but the level of phosphate incorporation reached only the level of the untreated HL-60 cells and thus fell far short of the level observed in these cells after PMA treatment. R-94 cells may thus contain a protein kinase C, which is also the phorbol diester receptor, that is not effective. Based on our previous studies, however, the acquired resistance of the HL-60 cell variant to phorbol diesters does not appear to result from an alteration in either the number of binding sites or binding affinity of the phorbol diesters to their receptor (protein kinase C) (17). These resistant cells, however, exhibit a reduced fluidity of the inner leaflet of the plasma membrane and/or of the cytosolic organellar membranes (42). Such change in membrane fluidity may thus play an important role in receptor mobility and affect crucial cellular events. It is reasonable to propose that the basis of the PMA resistance in these cells is a block in the signal transmission, required to initiate cell differentiation, that occurs subsequent to receptor occupancy. This signal transmission may involve the phosphorylation of some regulatory proteins in the nucleus, thus requiring the receptor to migrate to the nucleus. Our results with the proteins associated with the nucleus (P:3 and P:9), which show a lower degree of phosphorylation in the R-94 cells, are in support of such a suggestion. Alternatively, the signal may involve the phosphorylation of a protein or proteins that have to move from the location of the receptor to the appropriate target site in the cell nucleus.

Our results indicate that determining the changes in protein kinase C localization in different cellular compartments in the differentiation-susceptible and -resistant HL-60 cells after PMA treatment may prove fundamental in understanding how tumor promoters exert their pleiotropic biological effects in diverse cell types.

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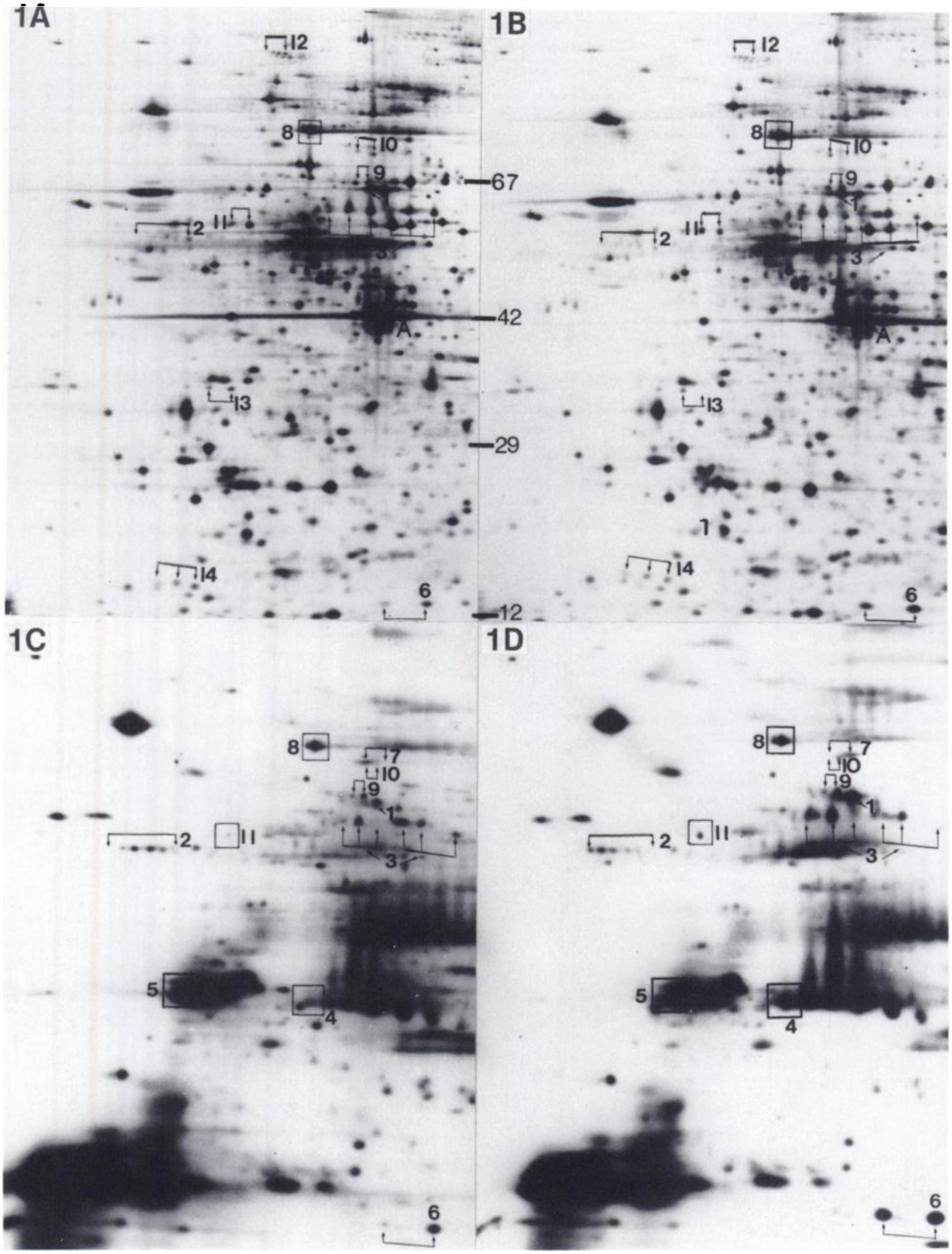


Fig. 1. Control HL-60 cells (*A* and *C*) or HL-60 cells treated with 10^{-7} M PMA (*B* and *D*), labeled with either [^{35}S]methionine (*A* and *B*) or [^{32}P]phosphate (*C* and *D*). Each panel shows the same section of the respective two-dimensional electrophoretic protein pattern, with acidic proteins to the *left* and higher molecular masses at the *top* (standard Cartesian representation). The *vertical scale* shows approximate molecular masses in kilodaltons. Areas of heavy streaking in phosphate-labeled patterns may represent nucleic acids migrating through the two-dimensional system (these areas are particularly prominent when Pharmalytes are used in the focusing gel). (P:13 lies just beneath a major nuclear phosphoprotein that obscures it on the ^{32}P-labeled pattern.) Due to variable phosphate and methionine content, some proteins are observed only on longer exposures with one of the labels. *A*, location of actin.

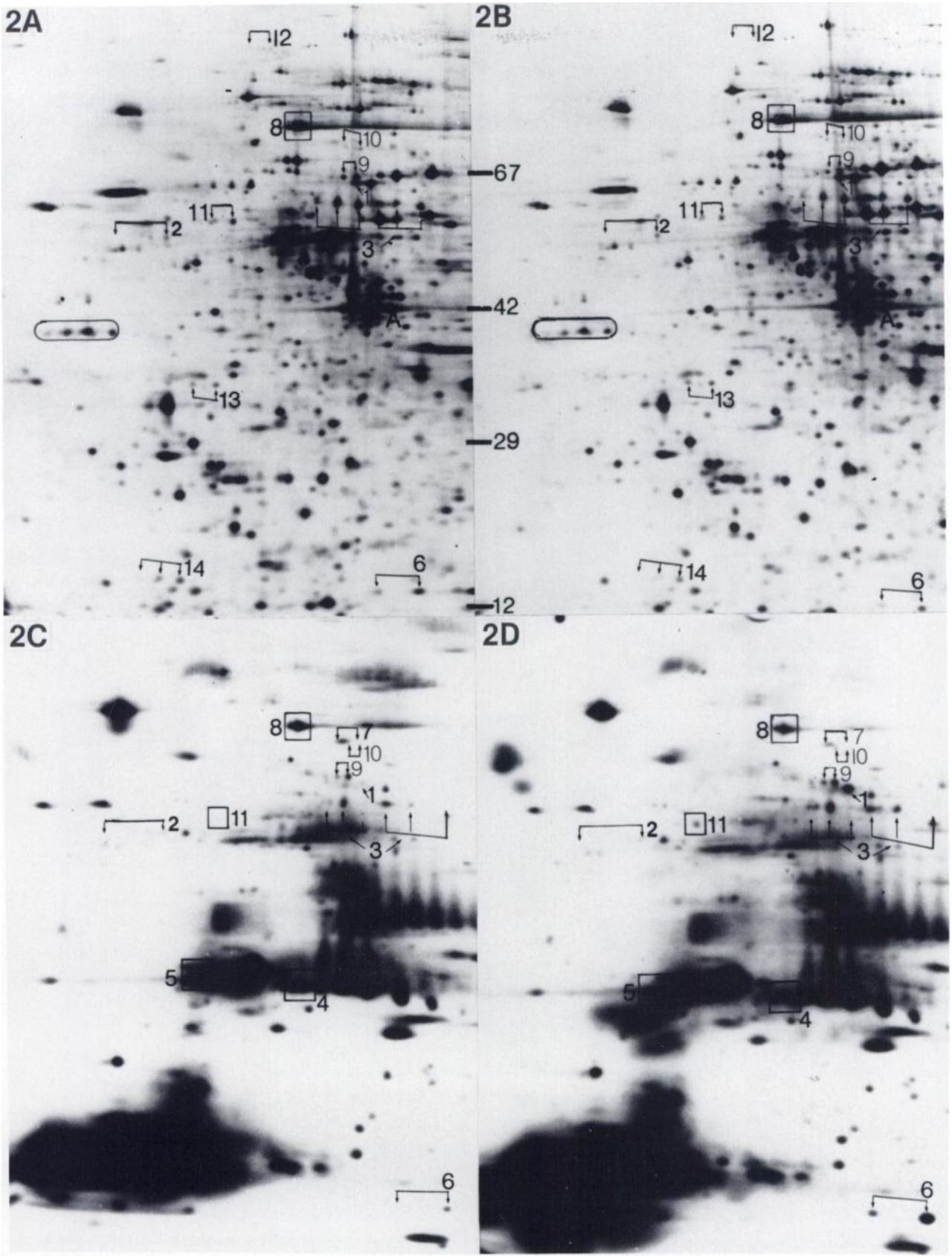
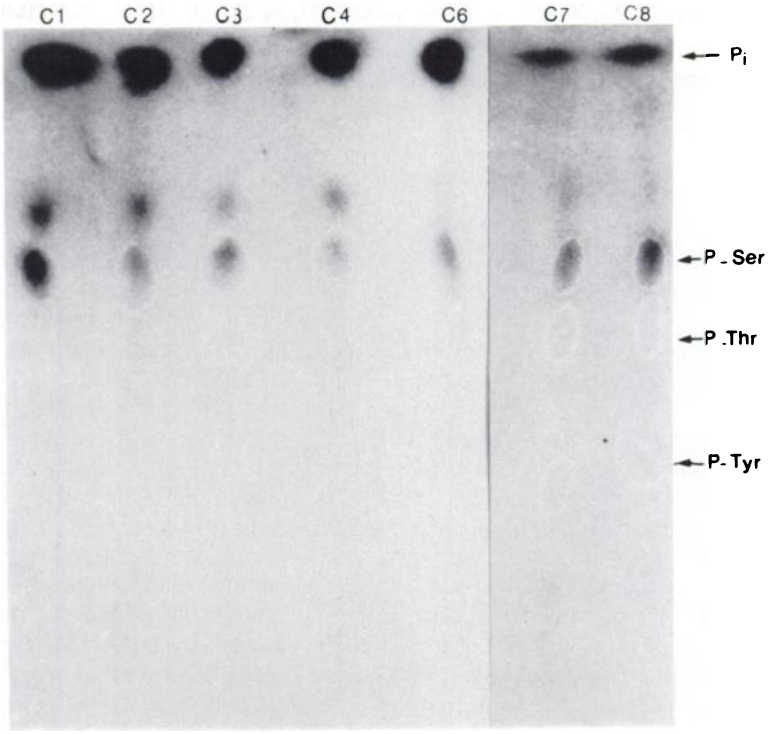


Fig. 2. R-94 cells treated as for Fig. 1.

3A



3B

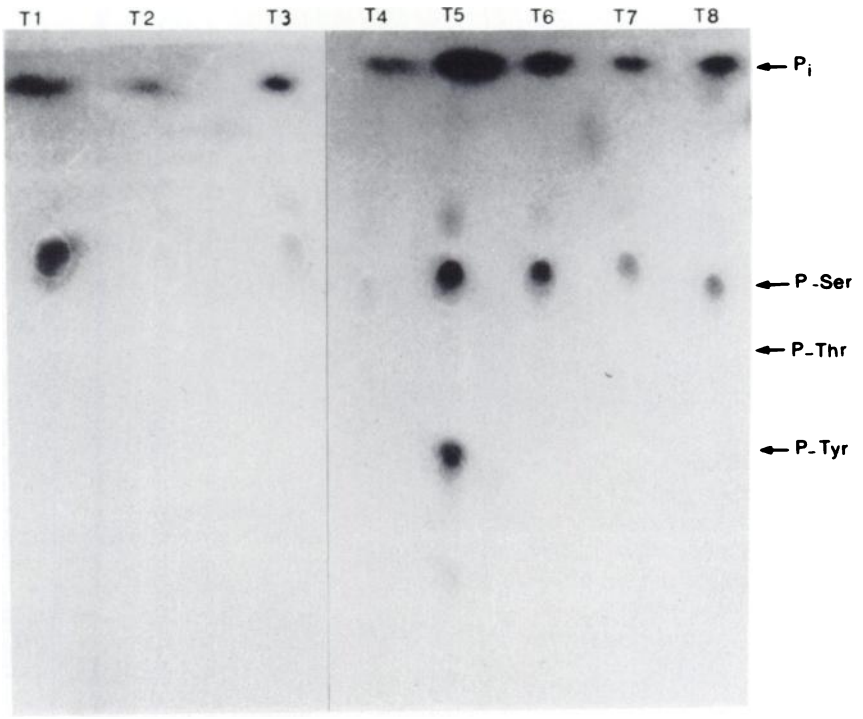


Fig. 3. Phosphoamino acid analysis of phosphoproteins excised from two-dimensional electrophoresis gels prepared from control (*A*) and PMA-treated (*B*) HL-60 cells. These gels were run using LKB ampholytes, which prevent streaking of PO_4-labeled material while yielding slightly lower resolution in the total protein ^{35}S-labeled pattern. *C1*, *C2*, *C3*, *C4*, *C6*, *C7*, and *C8* correspond to *T1*, *T2*, *T3*, *T4*, *T6*, *T7*, and *T8*, and the same number system in Fig. 1. Protein P:5 could not be reliably isolated and was therefore not included. Positions of the phosphoamino acid markers are indicated as follows: *P-Ser*, phosphoserine; *P-Thr*, phosphothreonine; and *P-Tyr*, phosphotyrosine. P_i, free phosphate.