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Comparative Analysis of p21 Proteins From Various Cell Types by Two-Dimensional Gel Electrophoresis

Nili Feuerstein and Iqbal Unnisa Ali

Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute (N.F.) and Laboratory of Tumor Immunology and Biology, National Cancer Institute (I.U.A.), National Institutes of Health, Bethesda, Maryland 20205

The products of the ras gene family are related proteins at a molecular weight of 21 kDa, designated p21. In the present study we used two-dimensional gel electrophoresis to compare p21 proteins from five different normal and malignant cell lines. Using a known protein (^{3}H-labeled translation initiation factor [eIF-4D]) as a standard internal marker for isoelectric point (pI), we show that p21 proteins from various cells differ only slightly in molecular weight (21–24 kDa) but express a wide variety in charge (pI 4.8 to 7) that could only be detected by the use of two-dimensional gel electrophoresis. p21 in NIH/3T3 cells was expressed as a single protein, which migrated at 21 kDa and pI 5.1. This peptide, which is probably the product of the normal cellular ras gene, was also detected in normal human lymphocytes. The synthesis of this peptide was not elevated in the transformed cells. However, transformation of NIH/3T3 fibroblasts and of human leukocytes was found to be associated with expression of qualitatively different forms of p21 peptides. Four additional p21-associated peptides of identical molecular weight (23 kDa), but multiple charge forms, were detected selectively in Kirsten murine sarcoma virus-transformed NIH/3T3 cells. Transformation of cells with Harvey murine sarcoma virus was found to be associated with prominent expression of two major pairs of p21-associated proteins, one at 21 kDa (pI, 5.2 and 5.3) and the other at 23 kDa (pI, 5.1 and 5.2). In HL-60 leukemic cells there was an additional, more acidic form (pI 5.0) of p21, which appeared to be absent or reduced in normal human lymphocytes. These results indicate that p21 from viral origin or cellular origin might be expressed in the cells in multiple charge forms. The capability to distinguish multiple forms of p21 and slight charge modifications associated with malignancy should call for the use of 2-D gel electrophoresis as an important tool in future studies involving p21 proteins.

Key words: p21, two-dimensional gel electrophoresis, leukemic cells, NIH/3T3 cells

Abbreviations used: Ha-MSV, Harvey murine sarcoma virus; Ki-MSV, Kirsten murine sarcoma virus; PMA, phorbol-12-myristate-13-acetate; 2-D gel electrophoresis, two-dimensional gel electrophoresis.

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The ras gene family (Ha-ras, Ki-ras, and N-ras) encodes proteins of 21–24 kDa, termed p21 [1–3]. The p21 proteins encoded by the ras genes of Harvey or Kirsten murine sarcoma virus (Ha-MSV, Ki-MSV) were shown to be responsible for the malignant transformation that is induced by these viruses [4,5]. Alteration in the normal cellular ras gene associated with substitution of a particular amino acid at position 12 or 61 was found in many human and rodent malignancies and was shown to induce the normal cellular gene to become activated [6–9]. Studies involving microinjection of p21 encoded by the activated form of the human Ha-ras gene or by the Balb-MSV ras gene provided further evidence for the direct transforming activity of this protein [10,11].

The p21 proteins are synthesized in the soluble fraction and subsequently migrate to the plasma membrane [12]. P21 was shown to bind guanine nucleotides [13] and have a GTPase activity [14]. The latter characteristic was found to be impaired in the protein encoded by the activated form of ras gene [14,15]. Certain posttranslational modifications were reported to occur in p21 proteins, such as autophosphorylation [13] and lipidation [16].

Posttranslational modifications, as well as mutations, might involve subtle changes in the charge of the protein without detectable alteration in its molecular weight. Detection of charge modifications in p21 proteins would require a method that will separate the proteins by both charge and molecular weight. In this study we present a standardized comparative 2-D gel electrophoresis analysis of p21 proteins in a variety of normal and malignant cellular systems. We show that p21 associated with either V-Ki-ras, V-Ha-ras, and probably N-ras could be distinguished by the use of 2-D gel electrophoresis and that p21 encoded by each of these ras genes is expressed in the cells as two or more charge forms. The capability to detect subtle charge modifications in the expression of p21 proteins and to distinguish between p21 from various origins suggests that 2-D gel electrophoresis might provide an important tool in studies involving p21 proteins.

MATERIALS AND METHODS

Cell Culture

NIH/3T3 cells and their transformants by Ki-MSV or by Ha-MSV [17] were kindly provided by Dr. Robert Bassin (National Institutes of Health, Bethesda, MD). HL-60 promyelocytic leukemic cells were cultured as previously described [18]. Human peripheral lymphocytes were purified from fresh blood as described [19] and incubated with phytohemagglutinin (Wellcome) 1 μg/ml for 24 hr.

Labeling of Cellular Proteins

HL-60 cells (5×10^6 cells/ml) and lymphocytes (10×10^6 cells/ml) were labeled with 150 μCi/ml of ^{3}H-leucine (Amersham) as previously described [18,19]. NIH/3T3 cells and their transformed derivatives were labeled [20] with ^{35}S-methionine (200 μCi/ml, 800 Ci/mmoles, New England Nuclear) for 18–24 hr at 37°C in DMEM containing 10% of the normal levels of methionine and 10% dialyzed fetal bovine serum. Following the labeling incubation the radioactive medium was removed, the cells were washed with phosphate-buffered saline, and lysed in buffer

containing 0.02 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, 0.005 M $MgCl_2$, 0.5% sodium deoxycholate, 1% (vol/vol) Triton X-100, and 2 mM phenylmethylsulfonyl fluoride. The cell lysate was then centrifuged at 100,000g for 30 min at 4°C. The supernatants were stored at −80°C until immunoprecipitation of p21.

Immunoprecipitation of p21

Immunoprecipitation was carried out by modification of the method described by Furth et al [20]. To 0.3 ml of the labeled cell extract (containing 10–20 × 10^6 trichloroacetic acid precipitable cpm) 7 μl of the monoclonal antibody (259, generous gift of Mark Furth) was added and the reaction was incubated overnight at 4°C. The immune complex was precipitated by the addition of 50 μl of a 10% (vol/vol) suspension of Formalin-fixed *Staphylococcus aureus* cells coated by antirat immunoglobulin serum for 2 hr at 4°C. The immunoprecipitates were collected by centrifugation and washed five times in a buffer containing 0.02 M Tris-hydrochloride, pH 8.8; 0.5% sodium deoxycholate; 0.5% Triton X-100; 0.15 M NaCl; 0.002 M EDTA; and 2 mM phenylmethylsulfonyl fluoride. The protein was eluted from the immunoprecipitates by boiling (3 min) in 20 μl of Laemmli sample buffer (0.0625 M Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol). The supernatant was then mixed with 2 volumes (40 μl) of electrofocusing dilution buffer (9.5 M urea; 2% ampholines, pH 3.5–10; 5% β-mercaptoethanol; 8% NP-40) and stored at −80°C until analysis by 2-D gel electrophoresis.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was done as previously described [21]. First-dimension isoelectrofocusing gels contained 2% LKB ampholytes (pH 3.5–10). Second-dimension gels were 12% acrylamide. Gels were fixed, stained, fluorographed, and dried as previously described [21]. Molecular weight standards (M_r = 14,000–92,000) were from BioRad. Fluorography was carried out with Kodak XAR-5 films at −80°C for 3 to 7 days.

Labeling the Marker Protein eIF-4D

Labeling of cells with ^{3}H-spermidine (31 Ci/mmole, New England Nuclear) was done as previously described [22]. HL-60 cells (2.5 × 10^6) were incubated with 2.5 μCi/ml of ^{3}H-spermidine for 24–48 hr in RPMI-1640 containing 10% dialyzed fetal bovine serum. Thereafter, the cells were spun down, washed, and lysed with isoelectrofocusing lysis solution (9.5 M urea; 2% NP-40; 5% 2-mercaptoethanol; 2% ampholines, pH 3.5–10). The lysate was spun at 2200 rpm for 5 min and the supernatant was stored at −80°C. Fractions of the ^{3}H-spermidine cell lysate (20 μl approximately 20 × 10^3 cpm) were mixed with the p21 immunoprecipitates prior to analysis by 2-D gel electrophoresis.

RESULTS

Analysis of the Electrophoretic Mobility of p21 by 2-D Gel Electrophoresis: Use of the ^{3}H-Spermidine-Labeled Translation Initiation Factor (eIF-4D) as a Marker Protein

To identify the migration of p21 in the pI dimension of 2-D gel electrophoresis, the immunoprecipitates of p21 (from HL-60 leukemic cells or NIH/3T3 cells) were

mixed with lysates of unlabeled cells. Following analysis by 2-D gel electrophoresis the gels were stained with Coomassie blue and exposed to fluorography. Superimposition of the fluorogram on the Coomassie blue-stained gel showed the position of p21 in reference to the pattern of the cellular Coomassie blue-stained proteins. It was seen that p21 in these cells migrated in an acidic position relative to actin, pI approximately 5.0–5.1 (data not shown). Furthermore, this analysis showed that the migration of p21 in the pI dimension (but not in the M_r dimension) is identical to the migration of a prominent Coomassie blue-stainable protein, which was identified [23] as the translation initiation factor eIF-4D (pI approximately 5.1, 18 kDa), suggesting the potential use of this protein as a marker for the migration of p21 on 2-D gel electrophoresis. eIF-4D has been shown to be a single protein in the cell that can incorporate labeled spermidine by formation of an unusual amino acid, hypusine, in a unique posttranslational modification [22,23]. Hence, to prepare labeled eIF-4D, we labeled cells with ^{3}H-spermidine as previously described [22,23]. The radiolabeled cell lysate that contained only one labeled protein, eIF-4D, was mixed with immunoprecipitates of p21 and analyzed by 2-D gel electrophoresis. Figure 1 demonstrates the electrophoretic migration of eIF-4D alone (Fig. 1A) or mixed with p21 immunoprecipitated from HL-60 cells (Fig. 1B). Apparently, the basic form of p21 in HL-60 cells migrates identically to eIF-4D in the pI dimension.

In further experiments the immunoprecipitates of p21 were routinely mixed with lysates of ^{3}H-spermidine-labeled cells prior to analysis by 2-D gel electrophoresis. Consequently, eIF-4D is detected as one of the radiolabeled proteins in all the fluorograms presented in this work. It provides a reference protein to analyze significant changes in the electrophoretic mobility of p21 polypeptides in various cellular systems, as will be further described.

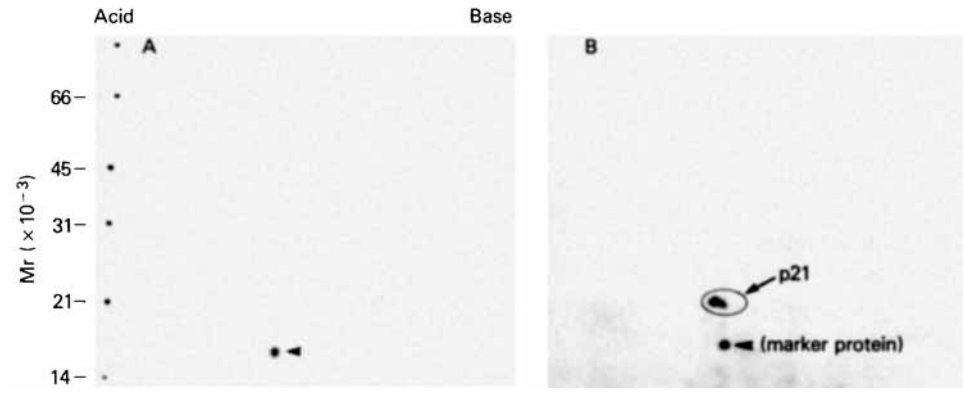

Fig. 1. 2-D gel electrophoresis analysis of p21 from HL-60 leukemic cells: Use of ^{3}H-eIF-4D as a marker protein. HL-60 cells (40×10^6) were labeled with ^{3}H-leucine as described. Thereafter, the cells were lysed and aliquots of approximately 150×10^6 CPM were used for immunoprecipitation. Labeling of the marker protein eIF-4D was done by incubation of HL-60 cells with ^{3}H-spermidine for 24 hr as described. The cells were then lysed with isoelectric focusing lysing solution. Fractions of the ^{3}H-spermidine-labeled lysate (approximately 20×10^3 CPM) were mixed with the immunoprecipitates and analyzed by 2-D gel electrophoresis. The gels were fixed, dried, and fluorographed. A) Lysate containing ^{3}H-eIF-4D. B) Lysate containing ^{3}H-eIF-4D mixed with immunoprecipitate of p21. Arrowhead, the marker protein eIF-4D.

Analysis of p21 Polypeptides in Fibroblasts Transformed by Ha-MSV and Ki-MSV

In further experiments we examined p21 proteins in virally transformed cells. NIH/3T3 cells transformed by Ha-MSV or by Ki-MSV were labeled with ^{35}S-methionine and lysed as described. Equal TCA-precipitable CPM (approximately 15 $\times$ 10^6) were used for immunoprecipitation of p21. Notably, several proteins were precipitated nonspecifically as they appear in control samples using normal rat serum, as well as in the p21 immunoprecipitates (Fig. 2). However, a group of several polypeptides at molecular weight ranges of 21–24 kDa were precipitated specifically with p21 antibody (Fig. 2B,D).

Peptide 1 (which expresses identical pI to the marker protein eIF-4D and was detected also in HL-60 cells) was precipitated in both Ha-MSV- and Ki-MSV-transformed cells. In addition to peptide 1 four other p21-associated peptides were differentially expressed in Ha-MSV- and in Ki-MSV-transformed cells. In cells transformed by Ha-MSV we detected two prominently synthesized pairs of peptides. One pair migrated at 21 kDa, pI of 5.2 and 5.3 (peptides 4 and 5, respectively). The other pair of peptides migrated at 23 kDa, pI 5.1 and 5.2 (peptides 6 and 7, respectively). While peptides 4, 5, 6, and 7 were not expressed in cells transformed by Ki-MSV, four other peptides (designated 8, 9, 10, and 11) at 23 kDa and pI range of 6 to 7 were specifically expressed only in the Ki-MSV-transformed cells. It should be noted that peptides 8, 9, 10, and 11 were not precipitated by Y13-238 anti-p21, which is specific for p21 encoded by Harvey ras [24]. On the other hand, Y13-238 anti-p21 precipitated 4, 5, 6, and 7, as well as peptide 1 in Ha-MSV-transformed cells (data not shown).

Comparison of p21 in Normal and Transformed Cells

Figure 3 demonstrates systematic comparison of p21 proteins in several normal and malignant cells. P21 proteins in NIH/3T3 cells transformed by Ha-MSV or Ki-MSV and in the leukemic cells, HL-60, were compared to p21 proteins in nontransformed NIH/3T3 cells and in normal human lymphocytes, respectively. As indicated in Figure 3, one peptide (see vertical broken line from the marker protein) is expressed in both the normal and the malignant cells. This peptide, which is designated as peptide 1 in the schematic presentation (Fig. 4), migrates at 21 kDa and has an identical pI to the marker protein (pI approximately 5.1). The fact that this peptide is expressed not only in the transformed cells but also in the normal cells suggests that it might be a product of a normal cellular ras gene. Notably, the amount of this protein is not elevated in the transformed cells. By contrast, peptides 4–7 were found to be expressed only in Ha-MSV-transformed NIH/3T3 cells (Fig. 2), and peptides 8–11 were expressed only in NIH/3T3 cells transformed by Ki-MSV. These peptides were not expressed in the nontransformed NIH/3T3 cells, and they were not expressed in HL-60 cells or in normal human lymphocytes, suggesting that these proteins might be specifically related to the viral Harvey and Kirsten ras genes, respectively.

Studies were performed to compare p21 in HL-60 leukemic cells, which carry an oncogene N-ras [25], to p21 in rapidly growing normal human lymphocytes. Lymphocytes were purified from peripheral blood as described [19] and incubated for 24 hr with phytohemagglutinin. Comparison of p21 in HL-60 cells (Fig. 3E) to this

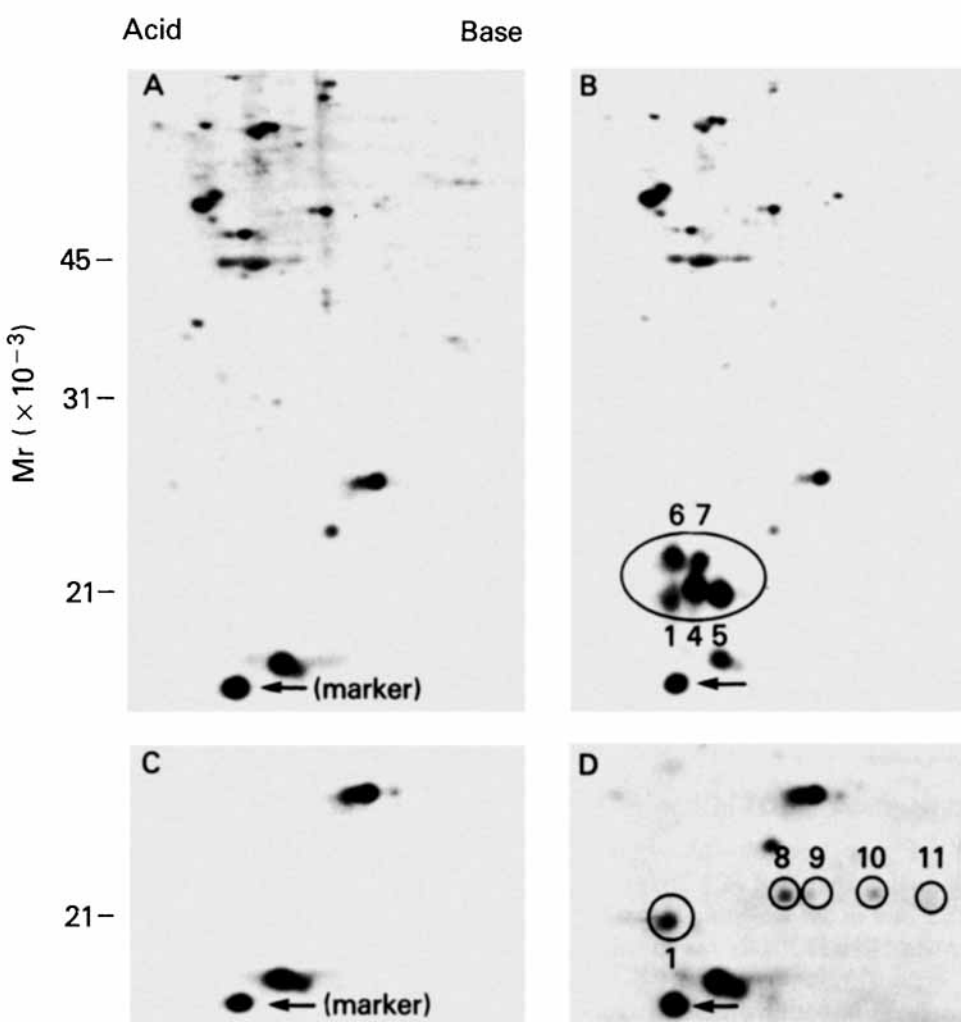

Fig. 2. Two-dimensional gel electrophoresis analysis of p21 immunoprecipitated from Ha-MSV-transformed NIH/3T3 cells and Ki-MSV-transformed NIH/3T3 cells. NIH/3T3 cells transformed by Ha-MSV or Ki-MSV were labeled with ^{35}S-methionine for 24 hr as described. Thereafter, the cells were lysed as described. Equal aliquots of TCA-precipitable CPM (approximately 15 × 10^6) were used for the immunoprecipitation assay. A) Ha-MSV-transformed cells: rat serum. B) Ha-MSV-transformed cells: p21 antibody. C) Ki-MSV-transformed cells: rat serum. D) Ki-MSV-transformed cells: p21 antibody. Arrows indicate the ^{3}H-marker protein. Circles and numbers indicate the peptides that were precipitated by the monoclonal antibody to p21.

in normal lymphocytes (Fig. 3D) suggests that peptide 1 is found in equal amounts in leukemic and normal cells, while peptide 2 (see schematic presentation in Fig. 4), which expresses a slightly more acidic charge compared to peptide 1, is prominent in HL-60 cells but absent or reduced in normal lymphocytes. Peptide 3, which appeared in both HL-60 cells and normal lymphocytes, was poorly resolved and its resolution was not reproducible in some experiments. Immunoprecipitation of p21 from human

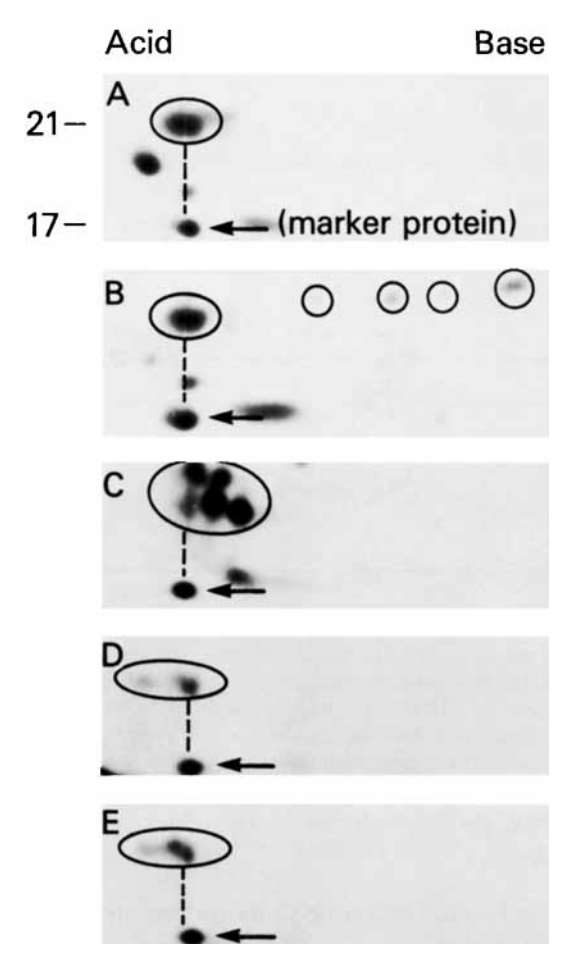

Fig. 3. Comparative 2-D gel electrophoresis analysis of p21 polypeptides in various normal and transformed cells. NIH/3T3 cells and their transformants by Ha-MSV and Ki-MSV were labeled with ^{35}S-methionine as described in Figure 2. HL-60 leukemic cells were labeled with ^{3}H-leucine as described in Figure 1. Human peripheral blood lymphocytes were purified as described [19] and then incubated with phytohemagglutinin (1 μg/ml) for 24 hr and labeled with ^{3}H-leucine as described. Equal aliquots of CPM of HL-60 cells and human lymphocytes or transformed and nontransformed NIH/3T3 cells were used for immunoprecipitation with anti-p21 antibody. All the immunoprecipitates were mixed with the lysate containing the ^{3}H-spermidine-labeled marker protein eIF-4D prior to analysis by 2-D gel electrophoresis. The panels show p21 immunoprecipitated from the following cells: A) nontransformed NIH/3T3 cells, B) NIH/3T3 cells transformed by Ki-MSV cells, C) NIH/3T3 cells transformed by Ha-MSV cells, D) human lymphocytes, and E) HL-60 cells. Arrows indicate the marker protein (eIf-4D). Circles indicate the proteins precipitated specifically by anti-p21 antibody in various cell systems. Broken vertical lines indicate the location of peptide 1 relative to the marker protein (see also schematic presentation in Fig. 4). Spots that are not circled are proteins that are precipitated nonspecifically.

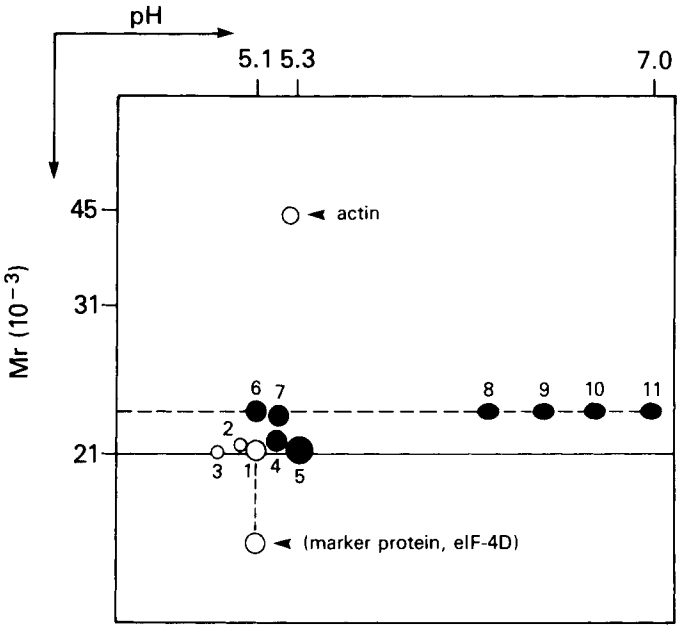

Fig. 4. Schematic presentation of p21 polypeptides in various cells as detected by 2-D gel electrophoresis. Arrowheads indicate the marker protein eIf-4D and actin. The numbers indicate p21 polypeptides as detected in various cells as follows: HL-60, peptides 1, 2, and 3; NIH/3T3 cells transformed by Ha-MSV, peptides 1, 4, 5, 6, and 7; NIH/3T3 cells transformed by Ki-MSV, peptides 1, 8, 9, 10, and 11; NIH/3T3 cells (nontransformed), peptide 1; human lymphocytes, peptides 1 and 3. (It should be noted that peptide 3 was poorly resolved, and its resolution was not reproducible in some experiments).

peripheral blood monocytes showed only a very faint spot that corresponded to peptide 1 (data not shown).

Comparison of p21 in HL-60 Leukemic Cells Before and After Differentiation Induced by Phorbol Ester

The promyelocytic leukemic cells, HL-60, undergo terminal differentiation upon exposure to phorbol ester (phorbol-12-myristate-13-acetate). This process involves arrest of cell growth and acquisition of morphological, cytochemical, and functional characteristics of macrophages [26]. We have previously studied early phosphorylation events and changes in synthesis of discrete proteins associated with differentiation of HL-60 cells induced by phorbol ester [18,19,27–31]. Since N-ras was reported to be one of the transforming genes in HL-60 cells [25], it was of interest to examine whether induction of growth arrest and differentiation of these cells is associated with any quantitative or qualitative change in the expression of the oncogene product, p21. To examine the effect of PMA treatment on the expression of p21 in HL-60 cells, we treated HL-60 cells with PMA (10^{-8}M) for 40 hr, thereafter the PMA-treated cells and the control cells were labeled with ^{3}H-leucine and lysed as described. Fractions containing equal CPM (approximately 150 $\times 10^{6}$) of PMA-treated cells and control cells were used for immunoprecipitation by anti-p21 antibody. Figure 5 shows that PMA treatment of HL-60 cells was not associated with any

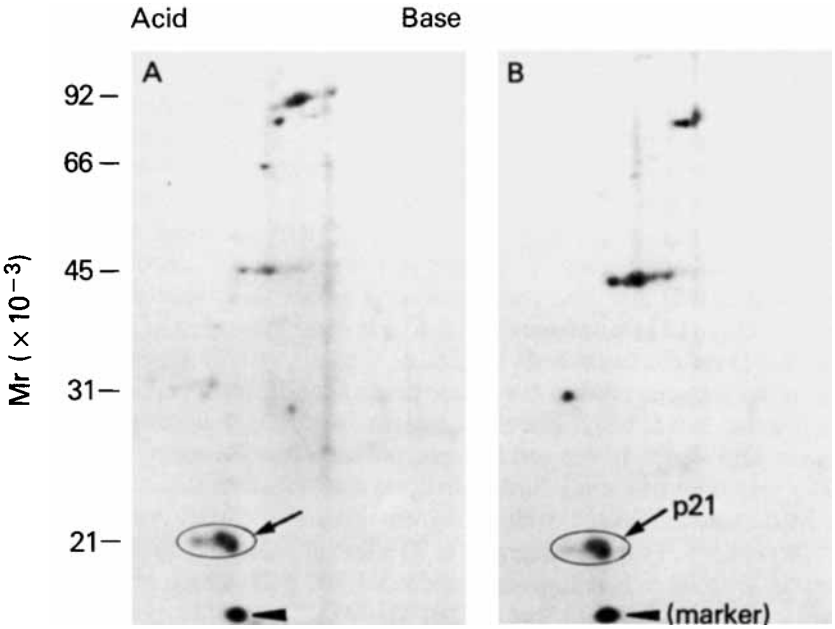

Fig. 5. Immunoprecipitation of p21 from HL-60 leukemic cells before and after differentiation by phorbol ester. HL-60 cells were exposed to phorbol ester 10^{-8} M for 40 hr as previously described [19,28]. Thereafter, the adherent cells (differentiated) as well as the control cells were labeled with ^{3}H-leucine as described. The cells were then lysed and equal fractions of CPM were used for immunoprecipitation. A) Immunoprecipitation of p21 in control HL-60 cells. B) Immunoprecipitation of p21 in phorbol ester-treated HL-60 cells. Arrowheads indicate the ^{3}H-marker protein (eIf-4D). Arrows indicate p21.

effect on the rate of synthesis of p21, and it was not associated with any qualitative change in the expression of the p21 polypeptides.

DISCUSSION

This work presents a comparative two-dimensional gel electrophoresis analysis of the ras-gene product, p21, in different cell lines. Using a standard protein marker we demonstrate the ability to detect qualitatively different forms of p21 proteins associated with malignant transformation of NIH/3T3 mouse fibroblasts and human leukocytes. We demonstrate that p21 from viral or cellular origin can be expressed in multiple forms that differ only slightly in molecular weight but display a wide variety in charge. We show that these multiple forms of p21 proteins could only be detected and specified by the use of 2-D gel electrophoresis.

Peptide 1 of p21 that migrated at 21 kDa and pI 5.1 (identical to the marker protein eIF-4D) was expressed in NIH/3T3 cells, normal lymphocytes, as well as in virally transformed NIH/3T3 cells and in the human leukemic cells, HL-60. It is, therefore, likely that peptide 1 represents the product of a normal cellular ras gene. It is also clear from Figure 3 that the synthesis of this protein is not elevated in malignant cells. By contrast, malignancy of NIH/3T3 fibroblasts and probably of human leukocytes appeared to be associated with expression of different forms of p21 peptides.

Transformation of NIH/3T3 by Ki-MSV was found to be associated with expression of four peptides immunologically associated with p21 that have identical molecular weight (approximately 23 kDa) but vary considerably in their charge (pI range of 6 to 7). Notably, the pI of these Ki-MSV-associated p21 proteins are markedly more basic compared to the peptide 1 of p21 (pI approximately 5.1) that was found in the nontransformed cells. A question of interest is what is the nature of the difference between the four proteins associated with p21 in Ki-MSV-transformed cells. It is possible that the difference in charge might be related to posttranslational modifications of p21 encoded by V-Ki-ras. Alternatively, some of these peptides might be immunologically associated with p21 but not directly encoded by V-Ki-ras. Previous studies [32] that used one-dimensional SDS-PAGE reported slower electrophoretic mobility (23 kDa) of activated C-Ki-ras when compared to normal C-Ki-ras. The use of 2-D gel electrophoresis enables us to show not only slower electrophoretic mobility of Ki-ras gene product but also to detect four different peptides distinguished by their charge. It will be of interest to compare by 2-D gel electrophoresis the gene products of activated C-Ki-ras and the gene products of V-Ki-ras.

The results of this study further indicate that transformation of NIH/3T3 cells by Ha-MSV was associated with prominent synthesis of two pairs of new p21-associated peptides. One pair migrates at 21 kDa (pI 5.2 and 5.3) and the other pair migrates at 22 to 23 kDa (pI approximately 5.1 and 5.2). These results corroborate previous studies that showed that p21 of Ha-MSV appears as two bands on one-dimensional SDS-PAGE [5]. Using 2-D gel electrophoresis we are now able to show that, in fact, each of the two p21 bands shown on one-dimensional gel electrophoresis consists of two distinct peptides that have identical molecular weight but vary in their charge.

Studies of p21 in lymphocytes showed that peptide 1, which was expressed in nontransformed NIH/3T3 cells, is also a major form of p21 in normal human lymphocytes and its synthesis is not elevated in HL-60 leukemic cells. However, in the leukemic cells, there was another form of p21 (peptide 2) that expresses an acidic shift in charge relative to peptide 1. This form of p21 appeared to be absent or reduced in normal lymphocytes. The possibility that this peptide might be related to the nature of malignancy in HL-60 leukemic cells (which were shown to contain an activated N-ras gene [25]) warrants further investigation. Peptide 3, which appeared in both HL-60 cells and normal lymphocytes, was poorly resolved and its resolution was not reproducible in some experiments. Our studies further showed that differentiation and growth arrest of HL-60 leukemic cells induced by PMA was not associated with any quantitative or qualitative change in the expression of p21, suggesting that the PMA-induced growth arrest in these leukemic cells does not involve an intervention in the expression of the ras-onc-gene.

In summary, the current work demonstrates the capability of 2-D gel electrophoresis to distinguish discretely between p21 proteins encoded by various ras genes and to detect subtle differences in charge of various p21 proteins associated with cellular transformation. We suggest that 2-D gel electrophoresis might provide an important tool in studies of p21 proteins.

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