Heterogeneity of Protein Phosphorylation in Metastatic Variants of B16 Melanoma

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ABSTRACT

The polypeptide and phosphoprotein profiles of a spectrum of B16 melanoma clones of defined metastatic potential have been analyzed by two-dimensional gel electrophoresis. To accommodate the documented instability of metastatic properties in B16 clones, in vitro biochemical assays were always accompanied by in vivo assays of the metastatic behavior using replicate samples of the same clonal populations harvested on the same day. To exclude differences in polypeptide and phosphoprotein profiles resulting from inherent variation in electrophoretic measurements made at different times, polypeptides and phosphoproteins were analyzed in unison for every clone, and a series of clones was examined in parallel in each experiment. Also, samples were electrophoresed simultaneously using a custom-designed apparatus capable of accommodating 20 two-dimensional samples. When tested under these stringent conditions, the polypeptide profiles of B16 clones were indistinguishable. Significant qualitative and quantitative differences in phosphoprotein expression were detected in each clone, but no correlations were found between alterations in protein phosphorylation and metastatic potential. Over 200 discrete phosphoproteins were detected in each clone, but interclonal variation was confined to approximately 10 to 15 phosphoproteins. Expression of three phosphoproteins with the following molecular weights (in kdaltons) and isoelectric points was strictly qualitative: pp96 (7.9); pp30 (8.2); and pp30 (8.8). In any given clone, they were present individually at equal intensities or were completely absent, but their expression was not coordinate. The data indicate that expression of polypeptide gene products is similar in B16 melanoma clones with widely differing metastatic abilities, but considerable clonal variability exists in posttranslational covalent modification of cell proteins. The possible contribution of protein phosphorylation and other posttranslational pathways in generating the extensive phenotypic heterogeneity observed in tumor cell subpopulations within the same tumor and in the rapid generation of new clonal variants with altered metastatic properties are discussed.

INTRODUCTION

Despite extensive investigation, identification of the cellular and subcellular alterations responsible for the aberrant behavior of tumor cells has remained elusive. If such correlations are ever to be made, it is becoming clear that more attention must be given to the source and the stability of the tumor cells used. To exclude differences in polypeptide and phosphoprotein profiles resulting from inherent variation in electrophoretic measurements made at different times, polypeptides and phosphoproteins were analyzed in unison for every clone, and a series of clones was examined in parallel in each experiment. Also, samples were electrophoresed simultaneously using a custom-designed apparatus capable of accommodating 20 two-dimensional samples. When tested under these stringent conditions, the polypeptide profiles of B16 clones were indistinguishable. Significant qualitative and quantitative differences in phosphoprotein expression were detected in each clone, but no correlations were found between alterations in protein phosphorylation and metastatic potential. Over 200 discrete phosphoproteins were detected in each clone, but interclonal variation was confined to approximately 10 to 15 phosphoproteins. Expression of three phosphoproteins with the following molecular weights (in kdaltons) and isoelectric points was strictly qualitative: pp96 (7.9); pp30 (8.2); and pp30 (8.8). In any given clone, they were present individually at equal intensities or were completely absent, but their expression was not coordinate. The data indicate that expression of polypeptide gene products is similar in B16 melanoma clones with widely differing metastatic abilities, but considerable clonal variability exists in posttranslational covalent modification of cell proteins. The possible contribution of protein phosphorylation and other posttranslational pathways in generating the extensive phenotypic heterogeneity observed in tumor cell subpopulations within the same tumor and in the rapid generation of new clonal variants with altered metastatic properties are discussed.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and the Laboratory Animal Services Division of Smith Kline & French Laboratories (Philadelphia, Pa.). Animals were age- and sex-matched within each experiment.

Cell Cultures and Cloning Procedures. The origin and properties of the B16 melanoma sublines, B16-F1 and B16-F10, have been described (9). Clones were isolated from each subline as described (25). Aliquots of each clone and the parent cell population from which they were derived were stored in liquid nitrogen for use as reference stocks. The metastatic properties of all clones were characterized within 6 weeks of their initial isolation.

Experimental Pulmonary Metastasis. Unanesthetized mice were inoculated i.v. via the tail vein with 5 × 10⁴ viable cells as a single-cell...
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suspension in 0.2 ml of PBS. Mice were killed 18 days later, and the lungs were removed, rinsed in water, and fixed in formalin. The number of lung tumor colonies (experimental metastases) was counted using a dissecting microscope (25).

Radioactive Labeling of B16 Cultures. B16 melanoma clones, suspended in DMEM plus 20% FCS (Grand Island Biological Co., Grand Island, N. Y.) were plated into 30-mm plastic tissue culture dishes at a cell density of 6 x 10^5 cells/dish. These cultures were incubated without a change of medium for 48 hr at 37° in a humidified chamber containing 5% CO₂. The semiconfluent cultures were rinsed 3 times with DMEM and replenished with 1.0 ml of DMEM plus 20% FCS. [³²P]Methionine (specific activity, 40 to 500 mCi/mmol; Amersham, Arlington Heights, Ill.), or [¹³C]methionine (specific activity, 8 mCi/ml; Amersham) was then added to each culture to give a final concentration of 150 μCi/ml and 1 mCi/ml, respectively. Following incubation for 18 hr at 37° the radiolabeled cultures were rinsed 3 times with 2 ml of Ca²⁺-, Mg²⁺-free PBS.

Extraction Procedures. Two hundred μl of NP40 extraction buffer (1% NP40, 10 mM NaCl, 10 mM Tris, 2 mM phenylmethylsulfonyl fluoride, and 1% Trasylol, pH 7.2) were added to each well followed by 24 ml of nuclease solution (14). After incubation on ice for 20 min, the cultures were scraped with a rubber policeman, transferred to thick-walled centrifuge tubes, and spun at 100,000 x g for 30 min at 4°. The supernatants were shelf-frozen using acetone-dry ice, lyophilized, and stored at −70°. Immediately before analysis by 2-D gel electrophoresis, the lyophilized detergent extracts were resuspended in 1 ml of sample reagent buffer (9.5 M urea, 5% 2-mercaptoethanol, 2% NP-40, 2% carrier ampholytes, 10 mM Tris, and 2% SDS, pH 6.8). The second dimension consisted of a 3% polyacrylamide stacking gel and a 10% resolving gel. Samples were electrophoresed at constant 6.8). The second dimension consisted of a 3% polyacrylamide stacking gel and a 10% resolving gel. Samples were electrophoresed at constant current (16 mA) for approximately 5 hr. Following electrophoresis, the gels were fixed, stained, and destained using conventional procedures (38).

Samples radiolabeled with [³²P]methionine were equilibrated with ENHANCE (New England Nuclear, Boston, Mass.) before drying. Autoradiography was performed using Kodak X-OMAT AR or Industrex AA film. Exposures were made at −20° or −70° for [³²P]methionine- and [¹³C]methionine-labeled samples, respectively. Comparative analyses of the polypeptide and phosphoprotein profiles were performed simultaneously by a 2-D gel electrophoresis system capable of resolving 20 samples concurrently. To confirm the reproducibility of the 2-D gel technique, the polypeptide and phosphoprotein profiles were compared in triplicate cultures of 2 B16 clones. Individual samples were handled and analyzed in independent experiments. Autoradiographs from replicate cultures were essentially superimposable, although minor quantitative differences were detectable (data not shown). Phosphoproteins were cataloged by their molecular weights (in kilodaltons) and isoelectric points.

Induction of Stress-induced Phosphoproteins. Cultures of B16-F1 were exposed to sodium arsenite (12 μM) in DMEM plus 10% FCS for 8 hr at 37°. [³²P] (1 mCi/ml) was added to the medium for the last 4 hr. Stressed and control (untreated) cultures were extracted in 1% NP40 and prepared for electrophoresis as described above.

RESULTS

Metastatic Phenotype of Clones Isolated from B16 Melanoma with Differing Metastatic Properties. A series of clones was isolated from the B16-F1 and B16-F10 melanoma cell lines and tested for their ability to form lung colonies when injected i.v. into C57BL/6 mice. A panel of clones showing a graded spectrum of metastatic abilities was selected for further study (Table 1).

Polypeptide Analysis of B16 Metastatic Variants. Confluent cultures of 6 B16 clones labeled with [³²P]methionine were extracted with 1% NP40 and analyzed by 2-D gel electrophoresis. Nearly 500 distinct polypeptides were resolved using this procedure. Visual inspection of the autoradiographs, including those overexposed to detect minor components, failed to reveal significant quantitative or qualitative differences among the

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>Formation of lung metastases by i.v. injection of clones isolated from B16 melanoma cell lines</td>
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<td>Single-cell suspensions (5 x 10⁶ cells) were injected i.v. in 0.2 ml of PBS into C57BL/6 mice, and lung metastases were counted 18 days later. The median values for each clone were derived from measurements on 6 mice with the exception of F10-C1, F1-C41, and F10-C123 which were derived from 8, 7, and 7 mice, respectively.</td>
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<tr>
<td><strong>Clone</strong></td>
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<tr>
<td>F1-C41</td>
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<td>F1-C14</td>
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<td>F10-C123</td>
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* Numbers in parentheses, range.

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<th>Table 2</th>
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<td>Principal phosphoproteins common to each B16 melanoma clone examined</td>
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<td>This comparison refers only to phosphoproteins soluble in 1% NP40.</td>
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<td><strong>Protein</strong></td>
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The abbreviations used are: PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM NaHPO₄, 7H₂O); DMEM, Dulbecco's modification of Eagle's minimum essential medium; FCS, fetal calf serum; NP40, Nonident P-40; 2-D, 2-dimensional; SDS, sodium dodecyl sulfate; 1-D, 1-dimensional.

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clones. However, a few minor dissimilarities were detected, but these could be attributed to experimental variations in the extraction procedures. A comparative analysis of the polypeptide profiles from all 6 B16 melanoma clones is shown in Fig. 1.

**Phosphoprotein Analysis of B16 Metastatic Variants.** Analysis of 1% NP40 extracts of 32P-labeled cultures on 2-D gels demonstrated the presence of approximately 200 phosphoproteins (Fig. 2). The most prominent of these, common to each B16 clone examined, are listed in Table 2. Considerable heterogeneity in the phosphoprotein profiles was identified, however, in individual clones. These differences were restricted to approximately 15 phosphoproteins (Fig. 3; Table 3). The most prominent differences centered on 3 phosphoproteins: pp96 (7.9); pp30 (8.2) and pp30 (8.8); pp96 (7.9) was detected only in one clone of very low metastatic potential (F1-C14), whereas pp30 (8.2) and pp30 (8.8) were found in the clone of the highest metastatic potential (F10-C123). However, these phosphoproteins do not represent specific markers for the low and high metastatic phenotype, respectively. For example, pp96 (7.9) was undetectable in 2 clones (F1-C41 and F10-C7) of low metastatic potential. In addition, in other experiments not performed in synchrony with the corporation of tightly bound phospholipid with specific proteins. The diversity of those reported here, we have failed to detect either pp30 (8.2) or pp30 (8.8) in B16 clones of high metastatic potential (data not shown). Several phosphoproteins of identical molecular weight (pp22) were readily detected in each B16 clone examined. The pattern of pp22 appearance suggested that each family of multiple spots represented a series of isoelectric variants (Fig. 3). However, the pattern of pp22 variants observed in different B16 clones was not indicative of metastatic potential. Indeed, there is no single group of phosphoproteins whose graded presence or absence correlates with the expression of the metastatic phenotype (Table 3).

Although the identity of the 12 most prominent phosphoproteins common to all B16 clones examined has not yet been established, they provide useful internal standards for analysis of each autoradiograph and can be used as markers for monitoring the consistency of the extraction procedures and the reproducibility of 2-D gels. As indicated in Fig. 2, all 12 components are major phosphoproteins the intensities of which among the B16 metastatic variants were remarkably constant.

**Induction of Stress-induced Phosphoproteins.** In a series of unrelated studies, we had previously observed that exposure of polyclonal B16-F1 cultures to sodium arsenite (12 μM) for 4 hr induced expression of 2 prominent phosphoproteins with molecular weights of 30,000 (Fig. 4). Reexamination of their migratory behavior on 2-D gels strongly suggested that these components were identical to pp30 (8.2) and pp30 (8.8) synthesized in un-stressed cultures of F10-C123 (see Fig. 3).

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### Table 3

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<thead>
<tr>
<th>Phosphoprotein (kDa)</th>
<th>Isoelectric point</th>
<th>B16 melanoma clone</th>
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<tr>
<td>pp30</td>
<td>7.9</td>
<td>++++</td>
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<tr>
<td>pp31</td>
<td>5.9</td>
<td>+++</td>
</tr>
<tr>
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<td>pp51</td>
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<td>pp55</td>
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<td>pp59</td>
<td>8.8</td>
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<tr>
<td>pp72</td>
<td>7.9</td>
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<tr>
<td>pp86</td>
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*+++ +++ ++ + ± ± ---, arbitrary values of densities of spots on the autoradiographs.

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The term "phosphoprotein" is used in its broadest sense. Cultures were labeled with 32P and subjected to nuclease digestion. Thus, radioactivity detected on the autoradiograph could be attributed to a number of protein modifications including, but not limited to, phosphorylation of specific amino acid residues, phosphorylation of the carbohydrate moieties of glycoproteins, deamidation, ADP ribosylation, and the incorporation of tightly bound phospholipid with specific proteins. The diversity of posttranslational modifications in which phosphate may participate (12, 16, 37) dictates caution in the interpretation of autoradiographs, and the term phosphoprotein, although convenient and widely used in describing this type of experiment, is used here with the above reservations.

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DISCUSSION

Previous studies on the protein composition of B16 melanoma cells using 1-D gel electrophoresis have failed to reveal any consistent quantitative or qualitative changes that correlate with metastatic ability (18, 22, 23, 31, 32). Nicolson et al. (20–22) have detected increased expression of a series of surface proteins in polyclonal B16 cell lines selected for their ability to localize in specific organs. However, expression of these molecules is considered to be related to their organ localization patterns rather than their metastatic potential, since other B16 cell lines with equal or greater metastatic capacities do not express these determinants (for review, see Ref. 22). In addition, inhibition of expression of these proteins induced by tunicamycin does not result in loss of metastatic capacity (19).

The present study was undertaken to determine whether the greater resolving power of 2-D gel electrophoresis might reveal differences undetected using 1-D methods. As emphasized in “Introduction,” in undertaking any experiment to correlate cellular properties measured in vitro with the metastatic behavior of cells in vivo, it is imperative that in vitro and in vivo observations be conducted in parallel and, if possible, be undertaken synchronously using replicate samples tested on the same day. This is particularly important in the search for biochemical correlates of metastatic ability. Since most, if not all, malignant tumors are heterogeneous and contain cellular subpopulations with diverse metastatic abilities (for reviews, see Refs. 10, 11, 27, and 28), isolation of clones of defined metastatic potential from the same parent population is needed to generate homogeneous material for comparative studies. Analysis of heterogeneous, polyclonal populations in which highly metastatic clones are a minority will not be adequate, since detection of any properties unique to these clones will be at risk of being obscured by the “background noise” of the nonmetastatic or weakly metastatic clones. Clonal analysis is complicated, however, by the finding that the metastatic phenotype is highly unstable in B16 melanoma clones. Significant drift in metastatic properties can occur within only a few weeks of the initial isolation of a clone (26). There is also evidence that the metastatic phenotype of certain B16 clones may be more labile than others. In addition, the metastatic potential of particular B16 clones may be perturbed by freezing and storing cells in liquid nitrogen and also by the composition of the medium in which they are cultured. To minimize these technical variables, the present study was conducted with clones that had been cultured in vitro for less than 10 weeks. In addition, replicate samples of the same cell populations used for biochemical analyses were injected in unison on all 6 clones, and the samples were electrophoresed simultaneously. Finally, replicate samples of the same clonal populations used for biochemical analyses were injected into mice at the same time to evaluate their metastatic behavior.

Using these stringent conditions, we have failed to detect any significant differences in the polypeptide profiles of individual clones, despite marked differences in their metastatic properties. The lack of overt differences is consistent with the findings mentioned above from studies using 1-D electrophoresis. For technical reasons, the present 2-D analysis is limited, however, to cellular constituents extracted by 1% NP40. Since most cytoskeletal (phospho)proteins are insoluble in this detergent, our conclusions apply principally to cytosolic and membrane proteins. Nonetheless, for the approximately 500 polypeptides in these categories detected in the present experiments, the data indicate that acquisition of the metastatic phenotype is not accompanied by gross deletion or overexpression of particular gene products. The minor differences that we did observe (Fig. 1) did not correlate with metastatic potential and represented polypeptides that, we know from other studies, are particularly labile and whose appearance or disappearance on 2-D gels is highly variable even among replicate extracts from identically treated cultures.

The possible relationship of protein phosphorylation to cell growth regulation has attracted considerable attention recently as a result of 2 sets of observations: (a) that several growth hormones, including epidermal growth factor, platelet-derived growth factor, and insulin, are believed to exert their action, at least in part, via phosphorylation of their membrane receptors (1, 6, 7, 33, 35); and (b) that the transforming gene products of several avian and mammalian retroviruses have been shown to be protein kinases (8, 15). Altered phosphorylation of cellular proteins induced by these gene products has been implicated in initiating the biochemical cascade that produces the pleiotropic cellular changes which accompany loss of growth regulation and acquisition of tumorigenicity (for reviews, see Refs. 2, 8, and 24). Nothing has been reported, however, about aberrant protein phosphorylation in metastatic cells.

Our experiments indicate that the pattern of protein phosphorylation in individual B16 melanoma clones is highly heterogeneous and, once again, no correlation can be made with metastatic behavior. This heterogeneity was not detectable, however, in conventional 1-D gel electrophoresis. Of the approximately 200 phosphoproteins detected in each clone, clonal variation was restricted primarily to the 10 to 15 phosphoproteins listed in Table 3. Three phosphoproteins were identified [pp96 (7.9); pp30 (8.2); and pp30 (8.8)] whose expression was strictly qualitative, with each being either present or absent in any given clone. However, their presence or absence was not coordinate.

The data on expression of these 3 phosphoproteins also emphasize the importance of screening a range of clones including comparison of several clones with similar metastatic properties when seeking biochemical correlates of metastatic ability. For example, had we compared only clones F1-C41 and F10-C123, we would have concluded erroneously that pp96 (7.9) and pp30 (8.2/8.8) were determinants unique to the low and high metastatic phenotypes, respectively. However, this is not the case, since these phosphoproteins are not expressed by other clones with similar metastatic properties (Table 3). Definition of a relationship between any putative biochemical marker and increasing metastatic potential thus demands comparison of an extended series of clones.

The tentative identification of pp30 (8.2) and pp30 (8.8) present in F10-C123 as "stress-induced" phosphoproteins is particularly intriguing. In a separate series of experiments we have been investigating the potential role of stress-induced proteins in augmenting the survival of blood-borne tumor cells. During these studies, we identified in the B16 melanoma (B16-F1) 2 conspicuous phosphoproteins, pp30 (8.2) and pp30 (8.8), in-
The principal conclusion of the present study is that the patterns of protein phosphorylation are highly heterogeneous in tumor cells clones with diverse metastatic abilities. This is in marked distinction to the uniformity of polypeptide expression observed in the same clones, and also contrasts sharply with the negative findings reported in several previous biochemical investigations that have attempted to identify molecular characteristics unique to B16 metastatic variants. Although we were unable to correlate specific changes in protein phosphorylation with metastatic potential, the heterogeneity of the phosphoprotein profiles suggests that these reactions may contribute to the phenotypic diversity of B16 clones in vitro and in vivo and thus may not be irrelevant to the expression of the metastatic phenotype. However, we cannot totally eliminate the possibility that the artificial conditions of cloning and in vitro culturing may have contributed to the heterogeneity of the phosphoprotein patterns. To minimize this possibility, clones derived from the parental B16-F10 and B16-F1 were used for experimentation within 10 weeks of their initial isolation. The effect of prolonged culturing of B16 clones in vitro on their phosphoprotein composition is currently being investigated.6

In addition to adding protein phosphorylation to the lengthy list of cellular properties that are expressed heterogeneously in subpopulations of tumor cells isolated from the same tumor or tumor cell line (10, 11, 27), posttranslational modification may also provide an explanation for the very rapid rate at which variants with altered metastatic properties are generated in clonal cultures of B16 melanoma (5, 20, 26, 29, 34) and other tumors (3, 4, 17, 35, 36, 39). The rate of formation of subclones with metastatic properties that differ significantly from the parent clone has been reported to be as high as 10-6 to 10-7/cell/generation (5, 17). Such frequencies are too high to be produced by genetic alterations and must thus arise via epigenetic mechanisms (5). Posttranslational modification of cellular proteins could provide such a mechanism.

Although the present study has focused on protein phosphorylation, other posttranslational events might be equally important in generating cellular diversity. Support for this view comes from studies showing that drug-induced alterations in the glycosylation of surface proteins in B16 melanoma cells produced reversible, yet significant, changes in their metastatic potential and their capacity to arrest and colonize specific organs (19).

In addition to intrinsic differences in the regulation of protein phosphorylation and other posttranslational modifications of cell proteins in different clones, posttranslational events of this kind may be particularly susceptible to alteration by factors in the cellular microenvironment resulting in further phenotypic change that will persist as long as or as briefly as the factor(s) in question is present. Accurate definition of the liability of specific phenotypic properties and a related understanding of the factors that can induce lability will therefore be essential if meaningful correlations are to be established between specific biochemical properties and tumor cell behavior in vivo. The present study indicates that, if we are to be successful in meeting this challenge, more attention must be given to the establishment of rigorous experimental protocols for the isolation and maintenance of tumor cell clones, for monitoring the stability of metastatic properties in clones during cultivation in vitro, and to undertake parallel in vitro and in vivo assays using replicate cell samples.

ACKNOWLEDGMENTS

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REFERENCES


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Fig. 1. 2-D gel analysis of polypeptides extracted from 6 B16 melanoma clones. Cultures of the B16 clones were labeled with [35S]methionine for 18 hr, extracted, lyophilized, and prepared for electrophoresis as described in the text. Approximately $1 \times 10^6$ cpm (10 µg protein) were loaded onto each gel. Apart from slight quantitative differences that are attributable to experimental variation (boxed areas), the polypeptide profiles of 6 B16 clones were indistinguishable. Isoelectric protein markers (pI) were: trypsinogen (9.30); lentil lectin variants (8.65, 8.45, and 8.15, respectively); myoglobin variants (7.35 and 6.85, respectively); human carbonic anhydrase B (6.55); bovine carbonic anhydrase B (6.55); β-lactoglobulin A (5.20); soybean trypsin inhibitor (4.55); and amyloglucosidase (3.50) (Pharmacia Fine Chemicals, Piscataway, N. J.). Molecular weight markers were analyzed in 2-D only: phosphorylase (M, 94,000); albumin (M, 67,000); ovalbumin (M, 43,000); carbonic anhydrase (M, 30,000); trypsin inhibitor (M, 20,100); and α-lactalbumin (M, 14,400) (Pharmacia Fine Chemicals).
Fig. 2. 2-D gel analysis of 1% NP-40 extracted B16 melanoma phosphoproteins. Cultures of B16 melanoma were labeled with [32P]orthophosphate for 18 hr and analyzed as described in Fig. 1. Approximately 5 x 10^6 cpm (10 μg protein) were loaded onto each gel. Isoelectric (pI) and molecular weight markers were as described (Fig. 1). The displayed autoradiograph is of F10-C4. The positions of the most prominent phosphoproteins (A-L) common to each B16 clone are indicated.
Fig. 3. Comparative 2-D electrophoresis analysis of the phosphoproteins in a panel of B16 melanoma clones of distinct metastatic potential. Cell extracts, sample preparation, and electrophoresis were performed as described in Fig. 2. Quadrants (Qd) of individual autoradiographs were compared for each B16 clone. For the sake of clarity, only 3 autoradiographs, from clones F1-C14, F10-C1, and F10-C123, are displayed. A summary of a complete comparison among the 6 B16 clones is presented in Table 3. Phosphoproteins differing significantly in their expression among the B16 clones were catalogued as: 1, pp96 (7.9); 2, pp93 (5.9); 3, pp88 (6.8); 4, pp76 (6.6); 5, pp75 (7.4); 6, pp73 (3.2); 7, pp66 (3.6); 8, pp54 (5.5); 9, pp54 (5.0); 10, pp32 (5.3); 11, pp30 (8.2); 12, pp30 (8.8); 13, p22 (bars, multiple spots. Boxed area in Qd 1, constellation of indiscrete spots whose composition was similar in each B16 clone examined except F10-C123. Although the clones displayed here exemplify the heterogeneity of expression of several of these phosphoproteins, in other clones, these differences were either enhanced or diminished (Table 3).
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