Secreted Phosphoprotein Markers for Neoplastic Transformation of Human Epithelial and Fibroblastic Cells

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ABSTRACT

A wide variety of rodent tumor cells of both fibroblastic and epithelial origins secrete a major transformation-related phosphoprotein with a molecular weight of approximately 62,000. Tumorigenic cells, regardless of the transforming agent, secrete 10-fold or more of this \( ^{32} \text{P} \)-labeled protein as compared with their nontumorigenic counterparts. In this study we have extended these previous findings to tumorigenic human cells of diverse origins (both sarcomas and carcinomas). Metabolic labeling of cells with \( ^{32} \text{P} \)orthophosphate and immunoprecipitation with antibody specifically directed against the rat transformation-dependent secreted phosphoprotein have been used to identify antigenically related human phosphoproteins (\( M_r \), 66,000–69,000). Of the 14 human cell lines examined, all 8 of the lethal tumorigenic cell populations secreted these phosphoproteins either in continuous culture or as fresh explants from nude mice while the six nonmalignant cell lines did not (tumorigenicity in all cases was assayed in nude mice). Included in our study were three tumorigenic human cell lines (two sarcomas, one carcinoma), each with a matched, nontumorigenic control. The very close correlation between secretion of these phosphoproteins and the tumor cell phenotype of both rodents and humans raises the possibility that they may be important for tumor growth in vivo.

INTRODUCTION

We have shown previously that a wide variety of virally and spontaneously transformed rodent fibroblasts secrete a major transformation-related phosphoprotein with a molecular weight, depending on the species of origin, of about 62,000 (1–3). Transformed fibroblasts secrete 10-fold or more of this major \( ^{32} \text{P} \)-labeled protein as compared with both their exponentially growing and their quiescent untransformed counterparts; therefore elevated secretion of this major \( ^{32} \text{P} \)-labeled protein is not simply linked to growth but instead is directly associated with transformation (1). Markedly elevated secretion of the \( M_r \), 62,000 \( ^{32} \text{P} \)-labeled protein is evident regardless of the transforming agent and is \( ^{32} \text{P} \) in a cell line that is \( ^{32} \text{P} \) for the transformed phenotype (1). This phosphoprotein is not antigenically related to \( \text{pp}60^{\text{src}} \) or \( \text{pp}60^{\text{v-src}} \) (2), and the fact that it is rapidly and efficiently released from cells following a pulse label (2, 3) also appears to distinguish it from viral oncoprotein products or cell protooncogene products of similar size (reviewed in Ref. 4).

More recently we have shown that elevated secretion (10-fold or more) of this major \( ^{32} \text{P} \)-labeled protein also identifies highly tumorigenic cells of epithelial origin and distinguishes neoplastic from hyperplastic epithelial cell populations (5). Hence unlike the classical phenotypic traits commonly associated with transformation of fibroblasts but that often do not accompany transformation of epithelial cells (see Refs. 6 to 8 for reviews), this secreted phosphoprotein marker identifies neoplastic cells of both fibroblastic and epithelial origins.

In the study described here, we have extended our prior observations with rodent cells to human cells and report that similar secreted phosphoprotein markers distinguish malignantly transformed human cells of both fibroblastic and epithelial origins from nonmalignant controls.

MATERIALS AND METHODS

Cell Lines and Cell Culture. All human cell lines (except for MNNG-T24) were obtained from the Animal Genetics and Production Branch, National Cancer Institute. Each cell line was tested in at least three animals; 5 x 10^6 cells were injected s.c. Completely consistent results were obtained with each cell line. In the case of the cell lines that resulted in progressively growing tumors, growth was evident by 14 days and continued steadily until it resulted in the death of the animals (usually within 2 months).

Purification of the Transformation-dependent \( M_r \), 62,000 Secreted Phosphoprotein from ts B77-Rat 1 Cells and Production of Antisera. The \( M_r \), 62,000 rat phosphoprotein was purified from the serum-free culture medium of ts B77-Rat 1 cells by a scheme partly based on our previously published method (3). Serum-free culture medium was passed over a DE-cellulose (Whatman DE23) column at pH 7.0; the column was subsequently washed with PBS and the phosphoprotein was eluted with a linear sodium chloride gradient in 10 mM phosphate, pH 7.0. The \( M_r \), 62,000 phosphoprotein elutes at approximately 0.4 mM NaCl. [Eluted fractions containing the phosphoprotein were identified by electrophoresis (see below).] The phosphoprotein fraction was dialyzed against PBS and further purified on a hydroxyapatite (BioRad) column equilibrated with PBS. The phosphoprotein was eluted from hydroxyapatite with a linear sodium phosphate gradient (0.01–0.50 mM, pH 7.0) in the range of 0.3–0.4 mM phosphate. At this point the \( M_r \), 62,000 phosphoprotein was at 90% or greater purity with only minor contaminants.
the M, 62,000 phosphoprotein was purified to apparent homogeneity on Laemmli (11) SDS-polyacrylamide gels. The gel was stained in 25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid with 0.025% (w/v) Coomassie Brilliant Blue R-250; the M, 62,000 band was cut out, homogenized in PBS and used for immunization of rabbits as described previously (2). Immunizing doses consisted of 200–400 μg of purified protein.

Metabolic Labeling, Immunoprecipitations, Slab Gel Electrophoresis, and Autoradiography. Metabolic labeling with 32P and L-[35S] methionine were as described previously (1, 2) but in serum-free media. To adjust for differences in cell number, pool sizes, uptake rates, etc., and to allow for direct comparisons between cell lines, volumes of labeled culture medium used for immunoprecipitations were normalized for total incorporation (trichloroacetic acid-precipitable dpm) in the cell layer. Differences in cell layer incorporation were 2-fold or less. Immunoprecipitations were carried out with rabbit antiserum against the M, 62,000 rat phosphoprotein; fixed Staphylococcus A (12) was used to absorb immune complexes and resulting pellets were washed three times with phosphate-buffered saline. Slab gel electrophoresis of reduced samples was according to the method of Laemmli (11); molecular weight markers used were bovine serum albumin (M, 67,000), catalase (M, 60,000), and ovalbumin (M, 43,000). Gels were 7.5% (w/v) acrylamide. Gels were dried and subjected to autoradiography with sensitized (13) Kodak X-omat AR film and a DuPont Cronex intensifying screen (for 32P) (14) at −70°C.

RESULTS

Identification of the Human Counterpart to the M, 62,000 Transformation-dependent Secreted Phosphoprotein of Rodent Cells. We began our study by examining a series of tumorigenic and nontumorigenic cell lines all derived from a single human osteogenic sarcoma cell line, HOS (15). The cell lines (all obtained from the American Type Culture Collection) and their tumorigenicities in nude mice are: HOS (15), not tumorigenic; MNNG-HOS (9, 15), chemically transformed HOS, tumorigenic and lethal; KHOS/NP (16, 17), Kirsten murine sarcoma virus-transformed HOS, nonproducer of virus, tumorigenic and lethal; and KHOS-321H (18), revertant of KHOS/NP, not tumorigenic. The tumorigenic potential of these cell lines was previously evaluated by the originators (15, 17), and we confirmed their observations (see "Materials and Methods"). Cells were labeled with 32P, and culture medium from the labeled cells was subjected to immunoprecipitation with four different antisera from rabbits immunized with the M, 62,000 transformation-dependent secreted phosphoprotein secreted by rat tumor cells (see "Materials and Methods"). Although all four rabbits produced antibody to the rat phosphoprotein, only two of the rabbits produced antibody that cross-reacted with a transformation-dependent phosphoprotein secreted by human cells (see Fig. 1). Several phosphoproteins present in culture media were nonspecifically precipitated by preimmune serum followed by Staphylococcus A, but phosphoproteins with molecular weights of 66,000–69,000 were specifically precipitated when immune serum was used. These 32P-labeled proteins were found in the culture media of the tumorigenic MNNG-HOS and KHOS/NP cell lines (Fig. 1, Lanes g and h) but not in the culture media of the nontumorigenic HOS or KHOS-321H lines (Fig. 1, Lanes i and j). In addition these 32P-labeled proteins are present in reduced amounts in the culture media of a MNNG-HOS cell population which, after continuous culture in our laboratory for 3 months, underwent a partial morphological reversion to a more normal phenotype (Fig. 1, Lane h). Passage of this partially reverted MNNG-HOS cell population in nude mice followed by explanting of the tumor cells to in vitro culture resulted in restoration of the original MNNG-HOS morphology (not shown) and markedly elevated secretion of the M, 66,000–69,000 32P-labeled proteins (Fig. 2).

Secretion of the Transformation-dependent Phosphoproteins Identifies a Variety of Malignantly Transformed Human Cell Populations. In addition to the HOS series of human cell
lines, we examined ten other human tumor cell lines for tumorigenicity and production of $M$, 66,000–69,000 phosphoproteins that cross-reacted with antibody raised to the $M$, 62,000 rat transformation-dependent secreted phosphoprotein. We found that six of these cell lines gave rise to progressively growing, lethal tumors in nude mice, two were not tumorigenic in nude mice, one gave rise to a small (0.4-cm) nodule that did not progress, and one gave rise to an approximately 1.2-cm-diameter tumor that subsequently regressed (see Table 1). The tumorigenicity of all of these cell lines was evaluated as described in "Materials and Methods"; histological examination of each of the lethal, progressively growing tumor types revealed that they were all invasive tumors. The fact that the T24 and J82 cell lines are not tumorigenic in nude mice has also been reported previously by others (19). The various cell lines were then analyzed for production of the $M$, 66,000–69,000 phosphoproteins and the results are presented in Table 1. (MNNG-HOS and HOS cells were included in the comparisons to serve as positive and negative controls, respectively.) Of the four cell lines that either were not tumorigenic or failed to produce lethal tumors (T24, J82, 5637, HT1376), none secreted detectable levels of the $M$, 66,000–69,000 phosphoproteins. Of the six cell lines that produced progressively growing, lethal tumors, three (HT1080, PA-1, and Wi Dr) secreted detectable levels of the $M$, 66,000–69,000 phosphoproteins. Of the six cell lines that produced progressively growing, lethal tumors, three (HT1080, PA-1, and Wi Dr) secreted the $M$, 66,000–69,000 phosphoproteins in culture (see Fig. 3 for comparison of HT1080 with HOS) while three (MNNG-T24, COLO 205, HeLa S3) did not. However, we have discovered that the three tumorigenic cell lines that did not secrete the phosphoproteins in culture did indeed secrete these phosphoproteins when freshly explanted from corresponding nude mouse tumors. In all three cases (MNNG-T24, COLO 205, HeLa S3), secretion of the phosphoproteins was restored by passaging the cells s.c. in nude mice. However, in all three cases, secretion of the phosphoproteins was lost soon after explant to in vitro culture. Fig. 4 displays a comparison of COLO 205 cells freshly explanted from a s.c. nude mouse tumor with COLO 205 cells that had been passaged in vivo and reestablished in culture. It should be noted that the $M$, 66,000–69,000 phosphoproteins

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**Table 1**

<table>
<thead>
<tr>
<th>Human &quot;tumor&quot; cell line and source</th>
<th>Tumorigenic in nude mice (5 x 10^6 cells s.c.)</th>
<th>Secretion of M, 66,000-69,000 phosphoproteins</th>
<th>Fresh explants from nude mouse</th>
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<tr>
<td>HOS-osteogenic sarcoma</td>
<td>(-)</td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>MNNG-HOS (chemically transformed HOS)</td>
<td>(+)</td>
<td>(+)</td>
<td>(−)</td>
</tr>
<tr>
<td>KHOS/NP (virally transformed HOS)</td>
<td>(+)</td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>KHOS-321H (revertant of KHOS/NP)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>5637 bladder</td>
<td>0.4-cm-diameter nodule does not progress</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>HT 1376 bladder</td>
<td>Grows and regresses</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>J82 bladder</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>T24 bladder</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>MNNG-T24 (chemically transformed T24)</td>
<td>(+)</td>
<td>(−)</td>
<td>(+)</td>
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<tr>
<td>HeLa S3 cervix</td>
<td>(+)</td>
<td>(−)</td>
<td>(+)</td>
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<td>COLO 205 colon</td>
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<tr>
<td>PA-1 ovarian</td>
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*Our unpublished data together with those of H. F. Dvorak.*
Fig. 4. Immunoprecipitates of culture media from $^{32}$P-labeled human COLO 205 cells cultured in vitro and in vivo. Immunoprecipitations and electrophoretic analysis as in Fig. 1. a, preimmune serum precipitate of medium from COLO 205 cells freshly removed from a s.c. nude mouse tumor; c, as in a but precipitated with immune serum; d, as in b but precipitated with immune serum. 67k, M, 67,000 position on gel.

Fig. 5. Metabolic labeling of the M, 62,000 (62k) transformation-dependent secreted phosphoprotein of rat cells with L-$^{35}$S]methionine; immunoprecipitations and electrophoretic analysis as in Fig. 1. F2408 is a "normal" established rat embryo fibroblast cell line and FRD4 is a transformed derivative of F2408. a, immune serum precipitation of medium from $^{32}$P-labeled FRD4 cells; b, preimmune serum precipitation of medium from $^{32}$P-labeled FRD4 cells; c, preimmune serum precipitation of medium from $^{32}$P-labeled F2408 cells; d, as in b but precipitated with immune serum; e, as in c but precipitated with immune serum. Note specific precipitation of M, 62,000 $^{35}$S]methionine-labeled band in Lane e that is absent in Lane c.

described here were not evident when unfrac tionated culture medium from $^{32}$P-labeled cells were analyzed by electrophoresis and autoradiography and that immunoprecipitation with specific antibody is required for their unequivocal detection. We have reported previously that several human tumor lines secrete a major M, 68,000 phosphoprotein (20). However, unlike the M, 66,000–69,000 phosphoproteins described in this report, the major M, 68,000 phosphoprotein does not react with antibody raised to the M, 62,000 transformation-dependent phosphoprotein of ts B77-Rat 1 cells; furthermore we have since found that it is secreted by a variety of normal and mortal human fibroblast cell lines (WI-38, WI-26, CCD-18 Lu, Detroit 551, 3260) in amounts comparable to tumorigenic cell lines. 5

Metabolic Labeling of the Transformation-dependent Secreted Phosphoprotein with L-$^{35}$S]Methionine. Our previously published results (1, 2, 5) as well as results presented here were derived from experiments based exclusively on metabolic labeling with $^{32}$P. Data obtained with $^{32}$P-labeling do not distinguish between the following: (a) tumorigenic cells secrete more of the transformation-dependent phosphoprotein than their normal counterparts; or (b) tumorigenic and normal cells secrete the phosphoprotein equally, but tumorigenic cells secrete the protein in a more highly phosphorylated form (10-fold or more). Metabolic labeling with L-$^{35}$S]methionine has been used to distinguish among these possibilities. Fig. 5 illustrates a typical experiment. Density equivalent cultures of F2408 (a rat fibroblast line) and FRD4 (a virally transformed derivative of F2408) were each labeled with $^{35}$S]methionine for 3.5 h. The culture media from these cells were then subjected to immunoprecipitation with antisera to the phosphoprotein and Staphylococcus A (volumes of culture medium were normalized to adjust for small $^{35}$S]methionine incorporation differences in the cell layers; see "Materials and Methods"). Several proteins were nonspecifically precipitated by this procedure, i.e., were present in both preimmune serum precipitates and immune serum precipitates, but only one protein was specifically precipitated by the anti-phosphoprotein serum (Fig. 5). Its electrophoretic mobility is identical to that of the M, 62,000 transformation-dependent secreted phosphoprotein, and it is visible in the culture medium of the viral transformant but not the normal fibroblast line. These results clearly indicate that these transformed cells actually secrete more of the transformation-dependent phosphoprotein than do their normal counterparts and that the differences observed with $^{32}$P-labeling are not simply attributable to differences in protein phosphorylation.

We have performed similar experiments with the human MNNG-HOS tumor cell line but have thus far been unable to detect the human M, 66,000–69,000 phosphoproteins with $^{35}$S]methionine labeling. We have had a similar experience with the hamster HSV-NIL8 (1) tumor cell line. Therefore we believe that, unlike the M, 62,000 transformation-dependent secreted phosphoprotein of rat cells, the corresponding antigenically related human and hamster phosphoproteins are either methionine poor or secreted in quantities that are low compared to that secreted by the rat tumor cells. Experiments involving metabolic labeling of these cell lines with other amino acids are in progress.

Comparisons between Transformation-dependent Secreted Phosphoproteins Described Here and Other Secreted Proteins of Similar Size. There are few known secreted proteins in this size range (M, 58,000–62,000 for rodents, M, 66,000–69,000 for humans) which are associated with the tumor cell phenotype. They include (to varying degrees) the plasminogen...
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activators (urokinase, \(M, 53,000-60,000\) (21-23), and tissue type plasminogen activator, \(M, 65,000-72,000\) (24, 25)) and \(\alpha\)-fetoprotein (26) which has a molecular weight in the range of 64,000-74,000. The phosphoproteins described here do not appear to be related to any of these proteins. Partially purified rat phosphoprotein (by DE-cellulose and hydroxylapatite chromatography; see "Materials and Methods") did not reveal any plasminogen activator activity in the presence of a urokinase chromogenic substrate (Kabi S-2444) nor did it display any activity in the fibrin-plasminogen overlay assay (27) (data not shown). The human phosphoproteins of MNNG-HOS origin were not immunoprecipitable with antibody directed against human tissue plasminogen activator (a gift of Dr. D. Collen); and our anti-phosphoprotein antibody, which binds the \(M, 62,000\) mouse transformation-dependent secreted phosphoprotein (3), did not bind mouse \(\alpha\)-fetoprotein (from Calbiochem) (data not shown).

DISCUSSION

With rabbit antiseras raised against the \(M, 62,000\) transformation-dependent secreted phosphoprotein of rat tumor cells, we have identified secreted phosphoproteins of similar size (\(M, 66,000-69,000\)) which are closely associated with the tumor phenotype of human cells. Of the eight human cell lines examined that produce progressively growing lethal tumors in nude mice, all secreted \(M, 66,000-69,000\) \(32P\)-labeled proteins that reacted with antibody to the \(M, 62,000\) transformation-dependent rat phosphoprotein. Five of the these lethal tumorigenic human cell lines secreted these phosphoproteins under in vitro culture conditions while three (COLO 205, MNNG-T24, HeLa S3) secreted the phosphoproteins only as freshly explanted cells from corresponding nude mouse tumors (Table 1; Fig. 4). Therefore it appears that these three tumorigenic cell lines undergo phenotypic alterations when grown in culture, at least with respect to secretion of these particular phosphoproteins. The remaining six human cell lines that we examined were either not tumorigenic in nude mice or failed to produce progressively growing lethal tumors. None of these cell lines secreted detectable levels of the antibody-reactive \(M, 66,000-69,000\) phosphoproteins (Table 1; Fig. 1).

Present among the 14 human cell lines that we examined are three sets of "matched pairs": two nontumorigenic cell lines and their corresponding tumorigenic derivatives (HOS, MNNG-HOS; T24, MNNG-T24); and one tumorigenic cell line and its nontumorigenic revertant (KHOS/NP, KHOS-321H). Direct comparisons were made between the related tumorigenic and nontumorigenic lines, and in all three cases we found that only the tumorigenic cell lines secreted the immunoprecipitable \(M, 66,000-69,000\) phosphoproteins (Fig. 1; Table 1). In addition, we examined a MNNG-HOS cell population which had undergone a morphological reversion during 3 months of continuous culture. This population of cells secreted the \(32P\)-labeled \(M, 66,000-69,000\) phosphoproteins in reduced quantity as compared with a MNNG-HOS population of original transformed morphology (Fig. 1). Injection of these cells s.c. into nude mice resulted in progressively growing tumors, and cells explanted (to tissue culture) from these tumors displayed the original MNNG-HOS morphology and markedly elevated secretion of the \(M, 66,000-69,000\) phosphoproteins (Fig. 2). Morphology and expression of the phosphoproteins was found to be stable over the course of 5 weeks following the explant; however, partial reversion was again evident by 6 weeks (data not shown).

In addition to undertaking an analysis of a variety of human cell lines by metabolic labeling with \(32P\), we have further investigated the expression of the transformation-dependent secreted phosphoproteins by metabolic labeling with \(L-[35S]methionine\). A "normal" established rat fibroblast line (F2408) and a transformed derivative (FRD4) were labeled with \([35S]\)methionine, and culture media were subjected to immunoprecipitation, gel electrophoresis, and autoradiography. Typical results have been presented in Fig. 5. Such experiments clearly establish that these transformed cells actually secrete more of the transformation-dependent phosphoprotein than their normal counterparts and that the differences observed by metabolic labeling with \(32P\) do not simply reflect differences in protein phosphorylation. Corresponding immunoprecipitations of cell lysates resulted in the precipitation of a \(35S\)-labeled \(M, 62,000\) protein from lysates of the transformant (FRD4) but not the "normal" F2408 cell line (not shown). Therefore in the case of this matched pair of cell lines and presumably for others as well, markedly elevated secretion of the phosphoprotein by the transformants appears to be a consequence of markedly elevated synthesis of the phosphoprotein. However, more complex explanations cannot as yet be ruled out (e.g., normal cells synthesize the phosphoproteins at levels equivalent to transformants but rapidly degrade it intracellularly).

In conclusion, there is a very strong correlation between elevated secretion of the \(32P\)-labeled proteins described here and the tumor cell phenotype. Not only does elevated secretion of these phosphoprotein markers correlate with tumorigenicity of a wide variety of rodent epithelial and fibroblastic cells (5), but it also correlates with the neoplastic phenotype of human cells derived from a diverse array of sarcomas and carcinomas, including fibrosarcoma, osteogenic sarcoma, bladder carcinoma, cervical carcinoma, colon carcinoma, and ovarian carcinoma. Hence unlike other protein markers such as carcinoembryonic antigen and \(\alpha\)-fetoprotein which are limited to particular carcinomas (26, 28), the phosphoprotein markers described in this report are associated with a wide variety of human tumor cell types. Furthermore the breadth of applicability of these human phosphoprotein markers is likely to extend even beyond the human tumor cell types examined in this study. For example, we have shown that the phosphoprotein markers do identify tumorigenic rodent cells derived from both breast and liver epithelium (5). The very close correlation between the secretion of these phosphoproteins and the tumor cell phenotype of rodents and humans raises the possibility that they may be important for tumor growth in vivo. We are continuing efforts to identify their function.

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REFERENCES

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