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Biochemistry

p53 transformation-related protein: Detection of an associated phosphotransferase activity

(protein kinase/p53 monoclonal antibody/transforming protein)

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ABSTRACT **Malignant cells of the mouse transformed by a variety of different agents have been found to express high levels of a 53,000 M_r phosphoprotein (designated p53). Little or no p53 can be detected in normal mouse cells. The nucleus appears to be the predominant site of p53 localization in transformed cells. p53-related antigens are also found in transformed cells of rat, hamster, rabbit, and human. In cells transformed by simian virus 40 (SV40), p53 forms a complex with SV40 tumor (T) antigen, resulting in the coprecipitation of T antigen by monoclonal p53 antibodies. Immune complexes of p53 precipitated from extracts of SV40- or methylcholanthrene-transformed cells by monoclonal p53 antibodies have protein kinase activity. This enzymatic activity is dependent upon divalent cations, utilizing Mn^{2+} more effectively than Mg^{2+}. The phosphorylation of p53 in this kinase reaction has been found to involve serine and threonine, but not tyrosine residues. In view of the finding that the transforming proteins of several different oncogenic viruses have kinase activity, the association of this activity with p53 is important with regard to the possibility of a common pathway of transformation by diverse agents.**

We have recently identified a protein having a molecular weight of 53,000, designated p53, which was found in a variety of transformed cell types of the mouse (1, 2). The antisera used to detect p53 by immunoprecipitation of [^{35}S]methionine-labeled cell extracts came from mice that had been hyperimmunized with syngeneic transplants of two independently derived BALB/c methylcholanthrene-induced sarcomas (Meth A and CMS4). The transformed mouse cells that were examined and found to express p53 included a series of chemically induced sarcomas, leukemias induced by x-ray or murine leukemia virus (MuLV), and cells transformed by either DNA- or RNA-containing tumor viruses. With one exception p53 was not found in normal mouse cells; cells derived from normal mouse thymus were the only ones having detectable levels of p53 (1). Parallel studies using sera from mice or hamsters bearing simian virus 40 (SV40)-induced tumors as the source of antibody have demonstrated a 53,000–55,000 M_r protein in SV40-transformed cells that precipitated as a complex with the viral tumor (T) antigen (3–8). These components, which have been called nonviral tumor (NVT) antigens, were shown to be phosphoproteins. Comparable components have also been demonstrated in cells transformed by Epstein–Barr virus (9) or Abelson MuLV (10).

Hybridomas secreting monoclonal antibody to p53 components of the mouse have now been isolated by three groups (10–12). The nucleus appeared to be the major site of localization, as indicated by immunofluorescence with two of these antibodies (11, 12), and both antibodies reacted with antigenically related components in the cells of other species.

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The present report represents further investigation with the p53 monoclonal antibody isolated by our group. With this specific serological probe, it has been possible to demonstrate that p53 has an associated kinase activity.

MATERIALS AND METHODS

Cells. The following cell lines were used. Methylcholanthrene-induced BALB/c sarcomas: Meth A and CMS4 (2). Nontransformed mouse cells: 3T3 (obtained from B. Peterkovsky). SV40-transformed cells: mouse 3T3WT, rat SVR-14B, hamster HE-WT, rabbit RK-WT, and human SV80.

Antisera and Monoclonal Antibody Detecting p53. These were: (*i*) BALB/c anti-CMS4 sera (2), (*ii*) BALB/c or Syrian hamster SV40 antitumor sera (13), (*iii*) BALB/c Schmidt–Ruppin avian sarcoma virus antitumor sera (14), (*iv*) p53 monoclonal antibody produced by a hybridoma (200-47) derived from a fusion between MOPC-21 NS/1 cells with spleen cells from a mouse immunized with CMS4 sarcoma cells (12).

Immunoprecipitation. All cell lines were grown in Eagle's minimal essential medium containing 10% fetal calf serum. Labeling with [^{35}S]methionine (50 μCi/ml) and [^{32}P]orthophosphate (0.5 mCi/ml) were for 2 hr (1 Ci = 3.7×10^{10} becquerels). Cells were lysed in RIPA buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% $NaDodSO_4$/0.5% Trasylol) and the resulting cell extracts were subjected to immunoprecipitation as described (14).

Protein Kinase Assay. Incubation of immune complexes with [γ-^{32}P]ATP (2000–3000 Ci/mmol) for 10 min at 30°C was essentially as described by Collett and Erikson (15, 16). The reaction products were analyzed in $NaDodSO_4$/10% polyacrylamide gels (17). The autoradiograms of dried gels were scanned in the Zeineh Soft Laser densitometer.

Analysis of Phosphorylated Amino Acids. Phosphoproteins were recovered from polyacrylamide gels and subjected to partial acid hydrolysis as described by Hunter and Sefton (18). ^{32}P-Labeled phosphorylated amino acids were identified by two-dimensional thin-layer electrophoresis/chromatography in the presence of phosphorylated amino acid markers. Phosphotyrosine was a kind gift of Karen Beemon.

RESULTS

Immunoprecipitation Reactions of p53 Antisera and p53 Monoclonal Antibody. The initial definition of p53 was dependent on antisera derived from two different sources: (*i*) mouse antisera prepared by hyperimmunization with Meth A or CMS4 methylcholanthrene-induced sarcomas (1, 2), and (*ii*) antisera from mice or hamsters bearing SV40-induced tumors (3, 4).

Abbreviations: SV40, simian virus 40; MuLV, murine leukemia virus; T antigen, tumor antigen.

More recently, a p53 monoclonal antibody has been derived from spleen cells of mice immunized with CMS4 sarcoma cells (12). Fig. 1 illustrates immunoprecipitation tests with labeled extracts of Meth A sarcoma using p53 antibody from these three sources. Antisera from mice growing SV40-induced tumors or Rous sarcoma virus-induced tumors were also found to precipitate a p53 component from Meth A sarcoma cells (data not shown).

Reactions of Monoclonal p53 Antibody with SV40-Transformed Cells of Different Species. SV40-transformed cell lines of mouse, rat, hamster, rabbit, and human origin were metabolically labeled with [^{32}P]orthophosphate and the resulting cell extracts were allowed to react with monoclonal p53 antibody in immunoprecipitation tests (Fig. 2). In each instance, a component ranging in molecular weight between 53,000 and 56,000 was precipitated. In certain cases, as with the human SV80 cells, the p53-like antigen appeared as a doublet, suggesting heterogeneity not only between species but also within a particular cell line. However, the presence of a single 55,000 M_r protein in the human SK-MEL-64 melanoma with the same monoclonal antibody (data not shown) indicates that the heterogeneity observed with SV80 cells is not a general characteristic of human transformed cells.

p53 monoclonal antibody also precipitated the SV40 T antigen from each of these transformed lines, indicating that the p53 component detected in the present study is related to the 53,000–55,000 M_r host protein with T antigen-binding activity reported by other investigators (3–8).

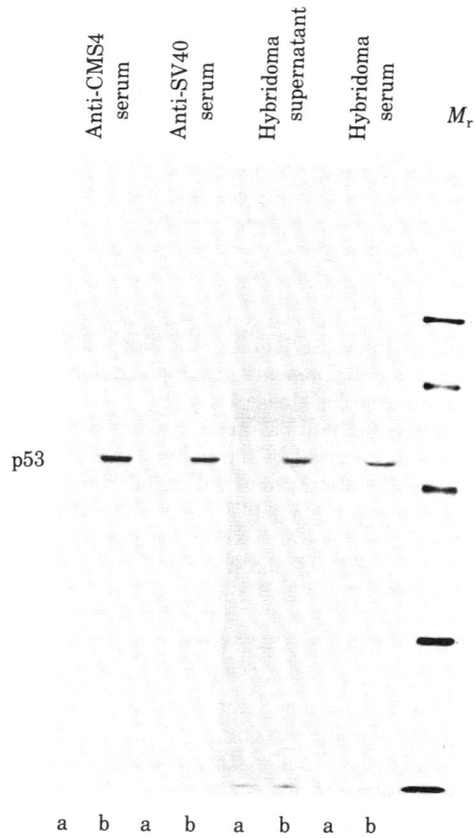

FIG. 1. Immunoprecipitation of p53 with antitumor sera and monoclonal antibody. Polyacrylamide gel analysis of [^{35}S]methionine-labeled proteins immunoprecipitated from extracts of Meth A sarcoma cells by the appropriate control sera (lanes a) or the indicated source of p53 antibody (lanes b). The molecular weight markers used were phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (45,000), carbonic anhydrase (30,000), and cytochrome *c* (12,300).

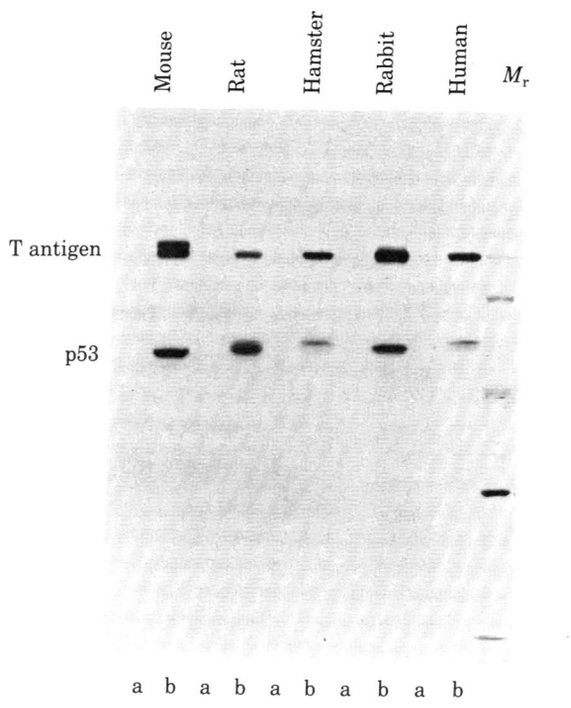

FIG. 2. Species distribution of p53-related antigens in SV40-transformed cells. Polyacrylamide gel analysis of ^{32}P-labeled proteins from extracts of SV40-transformed mouse (3T3WT), rat (SVR-14B), hamster (HE-WT), rabbit (RK-WT), and human (SV80) cells immunoprecipitated by control serum (lanes a) or p53 monoclonal antibody (lanes b). The molecular weight markers used were as in Fig. 1.

Association of a Phosphotransferase Activity with p53. Extracts of Meth A, 3T3, and 3T3WT (an SV40-transformed 3T3 cell line) were allowed to react with monoclonal p53 antibody, and the resulting immune complex was incubated with [γ-^{32}P]ATP to assay for protein kinase activity. p53 was selectively phosphorylated in immunoprecipitates of Meth A and 3T3WT cells; no p53 activity was detected in control 3T3 cells (Fig. 3). SV40 T antigen from 3T3WT cells was also phosphorylated. Although these findings suggest that p53 has phosphotransferase activity, it is not clear whether this activity is associated with, or is intrinsic to, p53. The fact that autophosphorylation of p53 can be detected in Meth A extracts indicates that this activity is independent of the presence of SV40 T antigen, which is separately phosphorylated in SV40-transformed cells, whether by the same or by a different enzymatic activity.

Characterization of the Phosphotransferase Activity. A plateau in kinase activity is reached after a fixed amount of p53 monoclonal antibody is added to increasing amounts of transformed cell extracts, showing that the immune complex kinase activity is saturable (Fig. 4*A*). Detection of phosphotransferase activity is absolutely dependent upon added divalent cations. The activity in the presence of 25 mM Mn^{2+} was at least 5-fold higher than that observed with 25 mM Mg^{2+} (Fig. 4*B*). This preference for Mn^{2+} over Mg^{2+} resembles the preferences previously reported for the $p60^{src}$ kinase of avian sarcoma virus (16) and the P120 kinase of Abelson MuLV (19).

Analysis of the Phosphorylated Proteins. Analysis of the phosphorylated amino acids of p53 and T antigens derived from 3T3WT cells is shown in Fig. 5. Two labeling procedures were used: (*i*) metabolic labeling: cells were exposed to [^{32}P]orthophosphate for 2 hr at 37°C followed by extraction and immunoprecipitation with p53 monoclonal antibody; (*ii*) *in vitro* labeling: extracts of cells were allowed to react with p53 monoclonal antibody, and the immune complex was incubated with

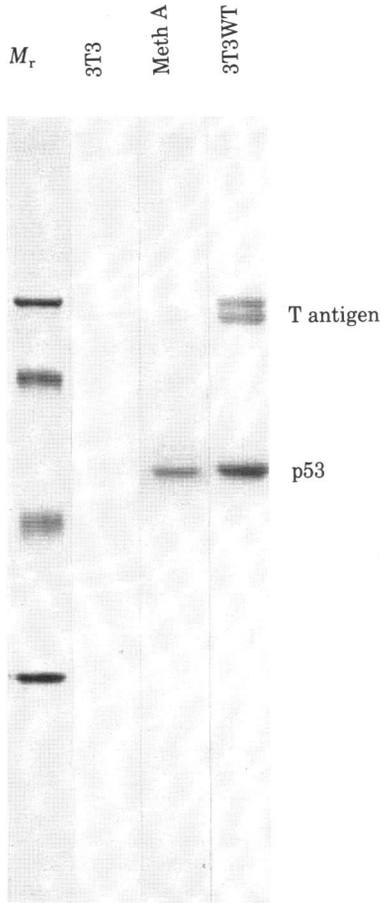

FIG. 3. Phosphorylation of p53 in immune complexes by incubation with [γ-^{32}P]ATP *in vitro*. Polyacrylamide gel analysis of immune complexes precipitated from extracts of 3T3, Meth A, and 3T3WT cells by the p53 monoclonal antibody and subsequently phosphorylated *in vitro* by incubation with 0.4 μM [γ-^{32}P]ATP and 25 mM Mn^{2+} for 10 min at 30°C. The molecular weight markers used were as in Fig. 1.

[γ-^{32}P]ATP for 10 min at 30°C. p53 from either 3T3WT or Meth A cells was found to be phosphorylated at serine residues when cells were incubated with [^{32}P]orthophosphate in culture. In the immune complex kinase assay, labeling of p53 with [γ-^{32}P]ATP was at both serine and threonine. No phosphotyrosine was evident in either labeling method. Phosphoserine and phosphothreonine were also demonstrable in T antigen that was labeled either metabolically with [^{32}P]orthophosphate or in an immune complex with [γ-^{32}P]ATP.

DISCUSSION

The cellular protein identified by the p53 monoclonal antibody used in our analysis has the following characteristics: (*i*) p53 appears to be a constant feature of tumor cells of the mouse, whether these were transformed by x-ray, chemicals, or DNA or RNA viruses or arose spontaneously (1, 2). In contrast, levels of p53 in normal mouse cells are either extremely low or undetectable. The one exception to this rule has been found with cells derived from normal thymus, in which p53 can be detected by immunoprecipitation of labeled cell extracts (1). Using SV40 tumor serum as the source of p53 antibody, Linzer and Levine (4) and Gurney *et al.* (11) have found low levels of p53 in nontransformed 3T3 cells. In contrast, the 3T3 lines we have tested have been negative for p53. (*ii*) Immunofluorescence studies have localized p53 to the nucleus of transformed mouse cells (12). Other p53-related reagents have also shown similar p53 localization (7, 11). Rotter *et al.* (10), using a rat monoclonal antibody to p53, have reported evidence for surface representation of this protein on mouse B cells and Abelson MuLV-transformed cells. Because we have used indirect immunofluorescence we have not been able to test the surface reactivity of our monoclonal p53 reagent on cells of B derivation. Preparation of a directly fluoresceinated p53 reagent should remedy this situation. (*iii*) p53-related components are found in a variety of species. In SV40-transformed cells, these components vary somewhat in molecular weight, ranging between 53,000 in mouse cells to 56,000 in human cells. In cells derived from a variety of human tumors, immunofluorescence tests with our monoclonal antibody to mouse p53 has revealed strong nuclear reactions (12). In addition, rapidly growing cultures of certain normal cells, such as early passages of kidney epithelium and fetal brain, were also found to express high levels of p53. In contrast to the persistence of p53 in malignant cells after cells reach confluence, p53 expression in the normal cell disappears upon contact inhibition of cell proliferation. (*iv*) In SV40-transformed cells, p53 forms a complex with T antigen, and this complex can be precipitated by either T antibody or p53 antibody (3, 11). The binding of p53 to T antigen has been found in SV40-transformed cells of a number of species. (*v*) Mouse and hamster p53 components are immunogenic in their species of origin. Tumors differ, however, in their capacity to induce p53 antibody. Immunization with two methylcholanthrene BALB/c sarcomas (Meth A and CMS4) resulted in p53 antibody production; comparable immunization with certain other similarly induced sarcomas did not. Although the basis for this difference is not known, higher levels of p53 in certain tumors, structural differences in p53 components, and adjuvant effect of p53-associated proteins (e.g., T antigen in SV40 tumors) may all be involved. (*vi*) In our original study with conventional p53 antisera, we found that p53 was present as a phosphoprotein (1). The p53 monoclonal antibody has now permitted the detection of a possible kinase activity for p53. Because this associated activity resulted in the autophosphorylation of p53, our ability to detect this activity with our monoclonal antibody must imply that both the active site of the kinase and the phosphoacceptor sites on the p53 molecule were conformationally accessible in the immune complex. Antibodies that block either site would fail to demonstrate the presence of autophosphorylation activity in the immune complex kinase assay (10).

Protein kinase activity has been associated with the transforming proteins encoded by a number of different oncogenic viruses. By using antisera directed against the avian sarcoma virus transforming protein p60^{src}, it was shown that p60^{src}-containing immune complexes have an associated phosphotransferase activity capable of phosphorylating the heavy chain of immune IgG (15). The transforming proteins of other retroviruses have similarly been shown to have an associated protein kinase activity, including the P120 antigen of the Abelson MuLV (19), the P140 antigen of the Fujinami sarcoma virus (20), the p90 of the Y73 sarcoma virus (21), the P115 antigen of the Gardner strain of feline sarcoma virus (22), as well as the P85 antigen of the Snyder–Theilen strain of feline sarcoma virus (23). In fact, the presence of this enzymatic activity is not unique to the transforming proteins of RNA-containing oncogenic viruses. The large T antigen of SV40 (24, 25), the middle T antigen of polyoma virus (26–28), and the tumor antigens of human adenoviruses 5 and 12 (29) have all been reported to have a similar associated activity. At least in the case of the SV40-transformed cells, there have been reports that the protein kinase activity can be separated from T antigen by conventional biochemical fractionations (24, 30). Because p53 is associated with T antigen

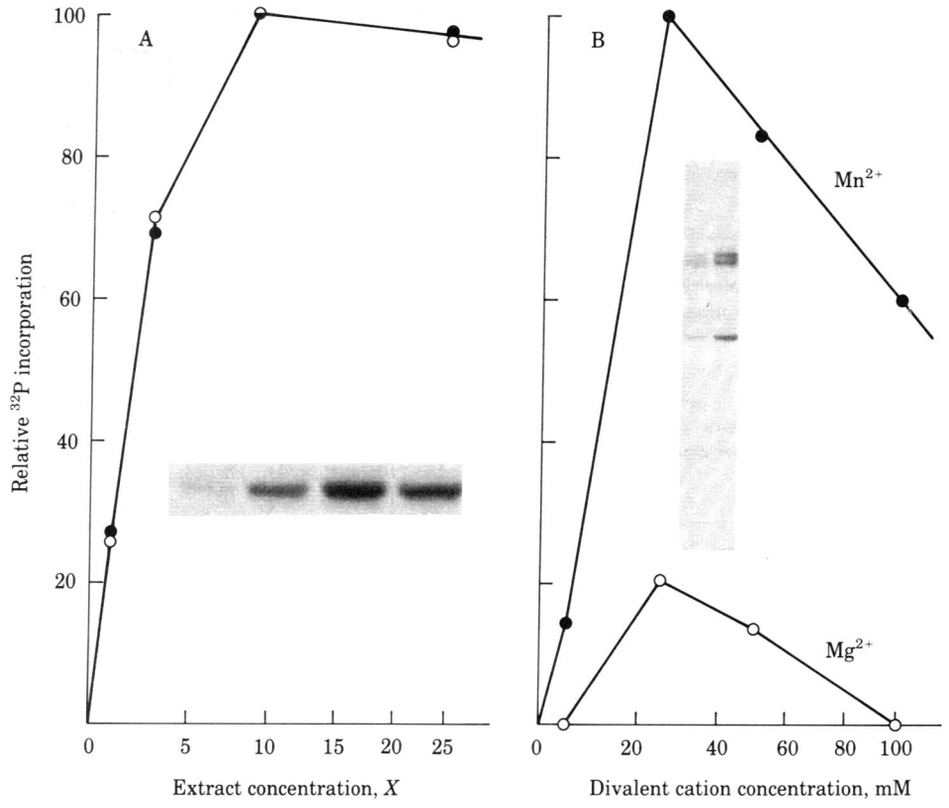

FIG. 4. Characterization of the p53 immune complex kinase activity. (*A*) Cell extract concentration dependence. Increasing concentrations of 3T3WT extract (1*X* = 0.15 mg of protein) were immunoprecipitated with a fixed amount (0.33 μl) of p53 monoclonal antibody (sera from hybridoma-bearing *nu/nu* mice). The resulting immune complexes were incubated with 0.4 μM [γ-^{32}P]ATP and 25 mM Mn^{2+}, before analysis on a 10% polyacrylamide gel. (*Inset*) Autoradiogram of the ^{32}P-labeled p53 component at each of the four extract concentrations tested. The extents of labeling of both p53 (○) and T antigen (●) at various extract concentrations were quantitated by densitometer tracing of the autoradiogram. (*B*) Divalent cation dependence. Various concentrations of either $MnCl_2$ (●) or $MgCl_2$ (○) were incubated (in the presence of 0.4 μM [γ-^{32}P]ATP) with immune complexes obtained with fixed amounts of 3T3WT extract (1.5 mg of protein) and p53 monoclonal antibody (0.33 μl of sera from hybridoma-bearing *nu/nu* mice). The extent of labeling of the p53 component, visualized by gel analysis, was quantitated by densitometric scanning of the autoradiogram. (*Inset*) Autoradiogram of proteins labeled in the presence of either 25 mM Mg^{2+} (left lane) or 25 mM Mn^{2+} (right lane).

in SV40-transformed cells, it is possible that p53 provides the associated kinase activity in this case.

With the exception of the p21 antigen of Harvey murine sarcoma virus (31), which autophosphorylates at a threonine residue, the transformation-related protein kinases associated with retroviruses have been found to catalyze the phosphorylation of tyrosines *in vitro* (18, 19, 21, 23). The occurrence of a similar kinase activity *in vivo* is suggested by the observation that there is an increase in the phosphotyrosine content of total cell protein upon transformation by either the avian sarcoma virus or the Abelson MuLV (32). However, this overall increase in cellular phosphotyrosine may not be a universal feature of transformation, because there is no detectable elevation in cells transformed by DNA viruses, including SV40, polyoma virus, and adenovirus (32). In fact, serines and threonines have been reported to act as acceptors for the kinases associated with the transforming proteins of both SV40 and adenovirus *in vitro* (25, 29). Our finding that the p53 kinase can phosphorylate at serines and threonines is, therefore, consistent with the possibility that T antigen may serve as a phosphoacceptor for p53 in SV40-transformed cells.

Given a protein having the characteristics of p53, what might its function be? A role in regulating cell division is perhaps the most interesting possibility and one that best fits the data presently available. In normal cells, initiation of cell division must be under stringent controls and mediators of this process are probably produced or activated only at some discrete point in the cell cycle and would not be allowed to accumulate to any degree. This would explain why p53 is generally not detectable in normal mouse cells, and why when it is found in rapidly dividing cells, such as normal human kidney epithelium, its expression correlates with the growth characteristics of the cells—i.e., high levels during the proliferative phase and disappearance after contact inhibition of cell division. In malignant cells, several types of genetic or epigenetic events could lead to a loss of this control over cell division, but in each case, the common element might be the constitutive expression of one or more regulators of the cell cycle. If p53 plays such a role, its persistent high levels in malignant cells could come about as a consequence of altered p53 regulatory genes (mutations, viral integration, etc.) or stable changes in the rate of p53 turnover. The binding of p53 by T antigen in SV40-transformed cells and possibly by transforming proteins of other RNA and DNA viruses may disrupt the ordinary pathways of p53 degradation and result in persistently high levels of functional p53. Among the questions that now need addressing is what proteins p53 interacts with in normal cells and in cells transformed spontaneously or by chemical or physical agents, because they may give clues into other components of the undoubtedly complex system of interacting proteins involved in the control of cell division.

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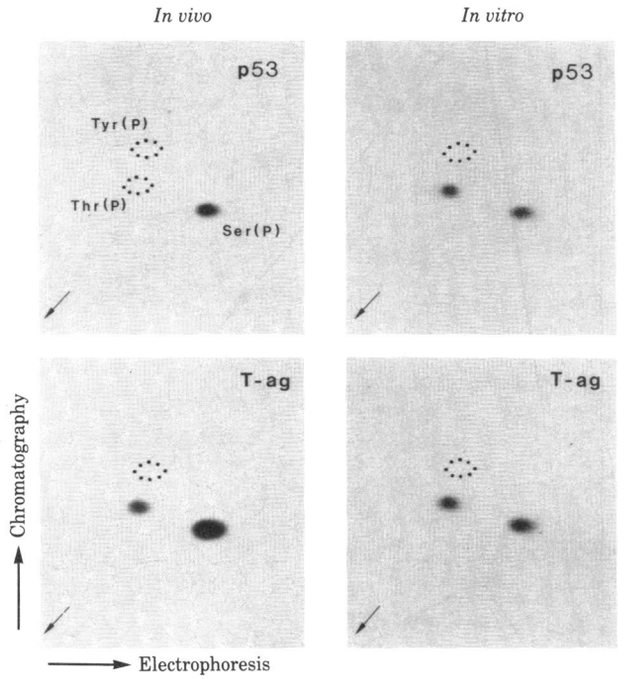

FIG. 5. Analysis of phosphorylated amino acids associated with p53 and T antigen (T-ag) from 3T3WT cells. T antigen and p53 radiolabeled either *in vivo* by incubation of 3T3WT cells with [^{32}P]orthophosphate or *in vitro* by incubation of immune complexes with [γ-^{32}P]ATP were fractionated by polyacrylamide gel electrophoresis. The appropriate protein bands were excised, eluted, and hydrolyzed with acid. The resulting phosphorylated amino acids were subjected to two-dimensional analysis as described by Hunter and Sefton (18). The origin is indicated with an arrow. Positions of stained marker phosphorylated amino acids are indicated by dotted outlines.

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