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Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase

(serine/threonine protein kinase/catalytically inactive kinase)

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ABSTRACT RNA-dependent protein kinase is a M_r 68,000 protein in human cells (p68 kinase) or a M_r 65,000 protein in murine cells (p65 kinase). p65/p68 is a serine/threonine kinase induced by interferon treatment and generally activated by double-stranded RNAs. Once activated, the known function of this kinase is inhibition of protein synthesis through phosphorylation of the eukaryotic initiation factor 2. Here we have investigated the potential for tumorigenicity in mice of murine NIH 3T3 clones expressing human p68 kinase, either the wild-type or a mutant inactive kinase with a single amino acid substitution in the invariant lysine-296 in the catalytic domain II. Expression of the mutant p68 kinase was correlated with a malignant transformation phenotype, giving rise to the production of large tumors of at least 1 cm in diameter within 7–12 days in all inoculated mice. In contrast, no tumor growth was observed for several weeks in mice inoculated with NIH 3T3 cell clones expressing either the wild-type recombinant p68 kinase or only the endogenous p65 kinase, the murine analogue of the p68 kinase. These results suggest that functional p65/p68 kinase (recently called PKR), by a still undefined mechanism, may also act as a tumor suppressor. Consequently, one of the pathways by which interferon inhibits tumor growth might be through its capacity to induce the enhanced expression of this kinase.

The double-stranded RNA (dsRNA)-activated protein kinase, recently called PKR for protein kinase RNA-dependent,§ is induced in mouse and human cells upon treatment with interferon (see refs. 1 and 2 for further references). An analogous enzyme has been described in rabbit reticulocytes, different mouse tissues, and human peripheral blood mononuclear cells (3–7). The human (p68) and murine (p65) kinases have been extensively characterized by biochemical studies, and their molecular cloning has been achieved (8–12). Interestingly, p65/p68 kinase shows a strong degree of sequence identity (38%) with the yeast GCN2 kinase, and both kinases manifest translational control through phosphorylation of eukaryotic protein-synthesis initiation factor 2 (eIF2) (12–15). In general, the p68 kinase is activated by binding to dsRNAs (10); however, some single-stranded RNAs, by virtue of their stem–loop structure, can bind and thus trigger activation (16, 17). The regulatory region(s) interacting with RNA has been localized at the N-terminal end of the kinase (12, 13, 18–20), upstream of the protein kinase catalytic subdomains (11). In addition to RNAs, the p68 kinase can also be activated by other polyanion molecules such as heparin, poly(L-glutamine), dextran sulfate, and chondroitin sulfate (21, 22). Once activated, this serine/threonine kinase is characterized by two distinct protein kinase activities in the presence of ATP: first, autophosphorylation and, second, phosphorylation of exogenous substrates (e.g., the α subunit of eIF2) (1). This latter activity prevents the recycling of phosphorylated eIF2-GDP to eIF2-GTP by the guanine-nucleotide exchange factor eIF2B. Thus phosphorylated eIF2 sequesters eIF2B and stops initiation of protein synthesis (23, 24). Through this mechanism, the protein kinase is implicated in the antiviral and antiproliferative effects of interferon. Direct evidence for this has recently been provided by the expression of the recombinant human kinase in murine cells and in *Saccharomyces cerevisiae*. Expression of the p68 kinase in murine cells mediated a partial resistance to encephalomyocarditis (EMC) virus growth (15), whereas in yeast, it mediated inhibition of cell growth (13). Activators of the p68 kinase in murine cells were probably the replicative intermediates of EMC viral RNA (15), and in yeast the activators were probably the dsRNA of fungal viruses that contaminate most laboratory strains of *S. cerevisiae* (13).

Our previous work has suggested that the lack of endogenous expression (with or without interferon treatment) and interferon induction of the p65/p68 kinase in different types of cells is somehow associated with tumor growth and antitumoral action (25–27). For example, malignant embryonal carcinoma stem cells do not express this kinase even after treatment with interferon (25). This is not due to the absence of interferon receptors on embryonal carcinoma cells since another interferon-induced enzyme, (2′-5′)oligoadenylate synthetase, is induced (25, 28, 29). In nude mice bearing human HeLa cell xenografts, treatment with species-specific human interferon β results in the induction of the p68 kinase in the tumor and a significant inhibition of its growth (26, 27).

To characterize the role of the p68 kinase in tumor growth or suppression, we investigated the tumorigenicity of murine NIH 3T3 cell clones expressing either the wild-type (active) or the mutant (inactive) form of the p68 kinase. Murine cells were used in this study since the activity of the transfected human p68 kinase could be assayed independently of the endogenous p65 kinase after immunoprecipitation with monoclonal antibodies (8, 30). We show that NIH 3T3 clones expressing inactive forms of the p68 kinase, produced by a point mutation of lysine-296 to arginine or to proline (18), initiate the development of rapidly growing tumors in nude

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Abbreviations: dsRNA, double-stranded RNA; eIF2, eukaryotic protein-synthesis initiation factor 2; EMC, encephalomyocarditis.

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§This serine/threonine protein kinase can be activated generally by dsRNAs and also by single-stranded RNAs and by heparin. In the literature, it has been referred to as dsRNA-activated protein kinase, P1/eIF2 kinase, DAI or dsI for dsRNA-activated inhibitor, and p68 (human) or p65 (murine) kinase.

mice. These results are in agreement with and extend those of Sonenberg and collaborators (31), who have recently reported the tumorigenic potential of NIH 3T3 cells expressing a deletion mutant of the p68 kinase.

MATERIALS AND METHODS

Plasmid Construction. The *Hin*dIII–*Eco*RI cDNA (2473 base pairs) encoding the p68 kinase was reconstructed in the vector Bluescript SK M13+ (Stratagene) using the K13 and R6 inserts isolated from a λgt11 expression library (11). The cDNA was then excised by *Hin*dIII–*Bam*HI digestion and inserted into the *Hin*dIII–*Bam*HI-cut pcDNA1/neo eukaryotic expression vector (Invitrogen, San Diego). The lysine-296 → proline (K296P) and lysine-296 → arginine (K296R) mutants¶ were generated by site-specific mutagenesis as described (18). Murine NIH 3T3 cells (plated in 60-mm dishes at 10^4 cells per dish) were transfected with 10 μg of the pcDNA1/neo vector carrying the wild-type kinase or each of the mutant p68 kinases by the calcium phosphate coprecipitation technique. After 4 hr of incubation, the cells were subjected to a glycerol shock for 2 min, washed once with complete medium, and cultured for 2 days. Cells were then cultured in medium containing G418 (Geneticin; GIBCO) at a concentration of 200 μg/ml for the first 2 days and then at 400 μg/ml. The surviving cells were collected by trypsinization and cloned in 24-well plates in growth medium containing 20% serum and G418 (400 μg/ml). Each transfection gave a yield of 10–15 drug-resistant clones. Some of these clones were included in the studies described here (see Tables 1 and 2). The detailed procedure for the isolation of the cell clones and their characterization has been described (15).

Mice. Congenitally athymic nude mice (*nu/nu*; 6-week-old females) on a Swiss background were from Iffa Credo (St. Germain sur l'Arbresle, France). Mice were maintained in a pathogen-free environment and were injected subcutaneously on the right side of the lower limb with 4 × 10^6 cells in Dulbecco's medium.

Culture of Tumor Cells. The tumors were recovered under sterile conditions and cleared of surrounding blood vessels and connective tissue. After washing extensively in phosphate-buffered saline, the tumor was cut into thin slices and homogenized by several strokes of a glass Dounce homogenizer. This homogenate was diluted with an equal volume of Dispase (Becton Dickinson) containing 0.5% collagenase (Worthington) and then incubated for 2 hr at 37°C before cells were recovered by filtering the homogenate by using a metal sieve. Cells were then washed once in culture medium (Dulbecco's medium containing 10% fetal calf serum and G418 at 400 μg/ml) before seeding the plates. Cells recovered from these tumors produced homogeneous cultures.

RESULTS

Murine Cell Clones Expressing Wild-Type Active or Catalytically Inactive p68 Kinase. NIH 3T3 cell clones were obtained by transfection with the expression vector pcDNA/neo containing cDNA encoding the p68 kinase (15) either as wild type (active kinase; ref. 11) or mutated at the invariant lysine-296 residue by a single change to arginine or proline (18). This lysine residue, located in catalytic subdomain II, is involved in the phosphate transfer reaction, and a single-point mutation at this residue completely inhibits kinase activity as was shown for a number of other kinases (reviewed in ref. 32). NIH 3T3 clones selected for the expression of pcDNA/neo alone were used as control cells. Fig. 1 shows

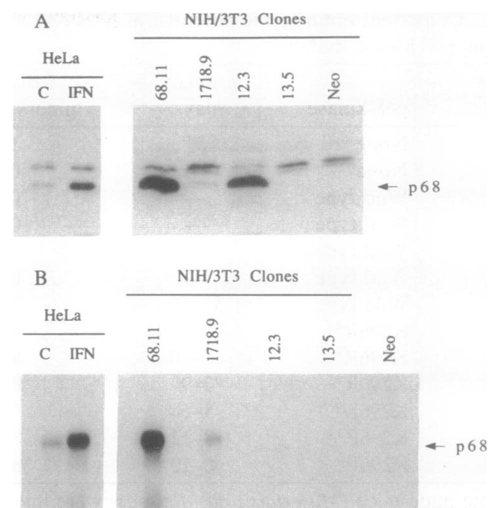

FIG. 1. Constitutive expression of the p68 kinase in NIH 3T3 clones by immunoblot assay. Extracts corresponding to 10^6 cells were analyzed by an immunoblot assay (*A*) using murine polyclonal antibodies raised against the purified p68 kinase (15, 33) and for protein kinase activity (*B*)—i.e., autophosphorylation of the p68 kinase after immunoprecipitation with specific monoclonal antibodies (15, 30). Autoradiograms are shown. HeLa cells treated with human interferon α at 500 units/ml (lane IFN) for 24 hr (34) were analyzed along with untreated control cells (lane C). NIH 3T3 clones 68.11 and 1718.9 were selected for expression of the wild-type human p68 kinase. Clones 12.3 and 13.5 were selected for the expression of the K296R and K296P mutants of p68 kinase, respectively. Clone Neo was selected for the expression of pcDNA1/neo alone. The protein kinase assay was carried out with [γ-^{32}P]ATP in the presence of poly(I)·poly(C) (as described in ref. 15) at 1 μg/ml. Note the lack of protein kinase activity of the mutant p68 kinase in clone 12.3.

the characterization of five different representative clones: two wild-type kinase-expressing clones (clones 68.11 and 1718.9); two mutant kinase-expressing clones, mutant K296R (clone 12.3) and K296P (clone 13.5); and one clone selected for the expression of pcDNA1/neo alone (clone Neo). As expected, p68 kinase activity (assayed by autophosphorylation) was observed only in clones 68.11 and 1718.9 (Fig. 1*B*). Different levels of the transfected p68 kinase (assayed by immunoblotting) were expressed (Fig. 1*A*). Clone 68.11 expressed severalfold higher levels of the wild-type transfected kinase compared to that induced by interferon in human cells (HeLa). On the other hand, clone 1718.9 expressed relatively lower levels of the transfected p68 kinase, and it was clearly detectable only after autophosphorylation. The mutant p68 kinase clone 12.3 expressed higher levels of the inactive kinase, whereas there was almost no expression in mutant clone 13.5. The latter was probably due to the loss of the transfected gene from the majority of cells during passaging, since clone 13.5 was initially selected for the expression of high levels of the mutant kinase (see below).

Consistent Tumorigenicity of Cells Expressing p68 Kinase Mutants. Athymic nude mice were injected with NIH 3T3 clones expressing either the wild-type kinase or the K296R and K296P mutant kinases. Mice injected with six independent NIH 3T3 clones expressing high levels of the different mutants of the p68 kinase produced large tumors in a relatively short time (Table 1). Consistently, at 5–12 days postinjection, the apparent diameter of the tumors was >5 mm and therefore clearly apparent. From this stage onward, the tumor mass increased very rapidly in few days, generating large-sized tumors. A mouse with a typical tumor is shown in Fig. 2. For example, for the tumor generated by clone 12.3, the mean tumor weight on day 14 was 949 ± 405 mg. Cells recovered from 12.3 tumors contained high levels of the

¶Lysine-296 corresponds to lysine-295 mentioned in ref. 11. This correction (ATCTAC starting at nucleotide 646 should be replaced by AATTCTAAC) was deposited in the GenBank (January 1991).

Table 1. Consistent tumorigenic potential of NIH 3T3 clones expressing p68 kinase mutants

NIH 3T3 clone	p68 kinase	Latency, days	Efficiency, tumors/injection
Neo	None	51	1/8
Neo	None	—	0/6
1718.9	Wild type	—	0/8
68.11.1	Wild type	—	0/8
68.9	Wild type	—	0/8
13.3	Wild type	—	0/8
68.11	Wild type	53–55	2/8
12.3	K296R*	7–9	8/8
12.1	K296R*	6–10	8/8
12.9	K296R*	7–9	8/8
12.31	K296R*	5–6	5/5
13.6	K296P*	8–12	8/8
13.7	K296P*	6–10	8/8

Athymic nude mice (*Materials and Methods*) were injected with the different NIH 3T3 clones expressing Neo alone, the wild-type p68 kinase, or the mutant (K296R and K296P) p68 kinases. The minimal time to produce apparent tumors of at least 5 mm in diameter was considered as the latency period. The observation period was 55 days.

*These clones expressed high levels of the mutant-transfected p68 kinase observed by immunoblot analysis as shown in Fig. 1.

mutant kinase, which were comparable to those found in cells before injection of mice (Fig. 3, lanes 12.3). Immunodetection of the p68 kinase with specific monoclonal antibodies in fixed cells indicated that cells recovered from a 12.3 tumor were homogeneous, since all cells were found to give an immunofluorescent signal (data not shown). It should be emphasized that with the six independent clones the tumorigenic potential of the mutant kinase-expressing clones was consistently 100% (Table 1). On the other hand, clones expressing the wild-type p68 kinase were not tumorigenic (Table 1). Among the five independent clones expressing the wild-type p68 kinase, no tumor growth was observed with four of the clones, whereas with the fifth clone, two out of eight injected mice developed tumors on days 53 and 55. Generation of tumors by the wild-type kinase-expressing cells, therefore, is a very rare event; furthermore, it occurs 6 weeks after the growth of rapidly growing tumors generated with mutant p68 kinase-expressing cells. A similar late tumor was observed in one out of eight mice injected with the clone Neo.

The lack of tumorigenicity of the wild-type kinase-expressing cells was not due to a constitutive production of interferon, since the levels of murine p65 kinase and the (2′-5′)oligoadenylate synthetase were found to be comparable to those levels in the corresponding cells expressing the mutant p68 kinase (15). Furthermore, injection of mice with a mixture of clones expressing the wild-type p68 kinase (clone 1718.9) and the K296R mutant (clone 12.3) did not modify the tumorigenic potential of mutant kinase-expressing cells (data not shown).

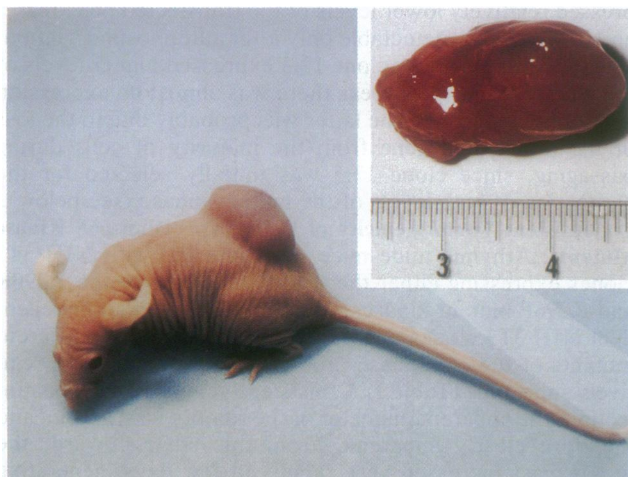

FIG. 2. NIH 3T3 clones expressing p68 kinase mutants produce large tumors in nude mice. A mouse was injected with clone 12.3. The picture was taken on day 14 after injection. (*Inset*) Tumor recovered from this mouse. The numbers on the ruler are in centimeters.

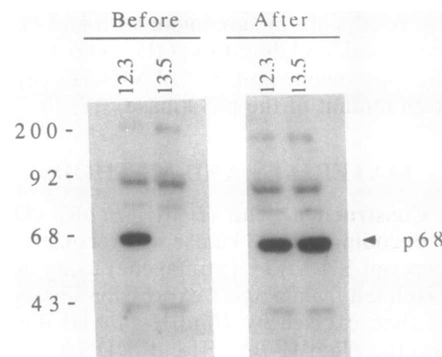

FIG. 3. Expression of human p68 kinase mutants in cells recovered from tumors developed in mice. Cells recovered from clone 12.3 and 13.5 tumors were cultured in Dulbecco's medium containing 10% fetal calf serum and G418 at 400 μg/ml. Extracts prepared 3 days later were assayed for the expression of the p68 kinase mutants by immunoblot analysis (as in Fig. 1). (*Left*) Samples of clone 12.3 or 13.5 cells at the time of injection into mice. (*Right*) Samples of tumor cells recovered from mice injected with clone 12.3 or 13.5 cells. An autoradiogram is shown. As was expected, no protein kinase activity was observed in these samples (data not shown).

***In Vivo* Selection of Tumorigenic Cells Expressing High Levels of the Mutant p68 Kinase.** Clone 13.5, initially selected for the expression of the K296P mutant p68 kinase, was found to express very little, if any, of the transfected gene (Fig. 1). Injection of mice with this clone resulted in the growth of tumors (of at least 5 mm in diameter) at 2–3 weeks after injection (Table 2). Consistent with the tumorigenic potential of the mutant p68 kinase, cells recovered from a typical 13.5 tumor were found to express high levels of the mutant p68 kinase (Fig. 3). These observations suggest that a few cells still expressing the mutant p68 kinase found in the clone 13.5 inoculum were specifically selected in mice because of their tumorigenic phenotype. Because of this selection, the appearance of tumors was slightly delayed to allow time for amplification. Interestingly, cells recovered from the clone 13.5 tumors had the capacity to induce rapidly growing large tumors within 5–7 days (Table 2).

To further demonstrate *in vivo* selection of cells expressing the mutant form of the p68 kinase, NIH 3T3 cells were transfected with the pcDNA/neo vector expressing the wild-type or the K296R mutant of the p68 kinase (*Materials and Methods*), and after 2 weeks, cells were injected into nude

Table 2. *In vivo* selection of tumorigenic cells expressing the mutant p68 kinase

Cells	Latency, days	Efficiency, tumors/injection
NIH 3T3 clone 13.5	14–22	8/8
13.5 tumor 1	5–6	5/5
13.5 tumor 2	5–7	5/5
13.5 tumor 3	5–7	5/5

Nude mice were injected with the clone 13.5 (*Materials and Methods*) initially selected for the expression of K296P mutant p68 kinase. Tumor growth became apparent in several mice at day 14 after injection, and subsequently all injected animals developed large tumors (1133 ± 624 mg) by day 28. Cells from three typical tumors were recovered (tumors 1, 2, and 3) and reinjected into mice.

mice. No tumor development was observed in mice injected with the cell population transfected with the wild-type kinase-expressing vector. On the other hand, four out of five mice injected with the cell population transfected with the mutant kinase-expressing vector developed tumors within 21–32 days (data not shown). As for clone 13.5, this delay in tumor development was probably due to the requirement for *in vivo* selection and amplification of mutant kinase-expressing cells.

Characterization of Late Tumors in Mice Injected with Clone 68.11 Expressing Wild-Type p68 Kinase. To further investigate the development of late tumors, mice were injected with the wild-type kinase-expressing clones 68.11 and 1718.9 and were kept under observation for 70 days. No tumor development was observed for clone 1718.9, which expressed low levels of functional p68 kinase (Fig. 1). On the other hand, for clone 68.11, which expressed high levels of functional p68 kinase (Fig. 1), two out of eight mice appeared to have small tumors on days 50–54 after injection (i.e., >5 weeks after the appearance of similar tumors in mice injected with mutant p68 kinase-expressing clones). Although several factors (not necessarily related to the p68 kinase) may be involved in the generation of such late tumors, it may as well be possible that prolonged periods of incubation in mice provide sufficient time for mutation of the wild-type p68 kinase and thus generate tumorigenic cells. In agreement with this latter possibility, cells recovered from late tumors were found to express the p68 kinase protein, but it had almost no protein kinase activity (Fig. 4). Accordingly, these tumor cells, when injected into mice, resulted in the development of large tumors (four out of four mice) in 7 days (data not shown). Thus, naturally occurring mutations of the wild-type kinase may occur in mice and be responsible for tumorigenesis.

Growth Properties of the NIH 3T3 Clones in Culture. In contrast to the striking effect of the mutant kinase in favoring tumorigenesis *in vivo*, no apparent differences in the morphology or in the cell cycle were observed in *in vitro* cell cultures of the wild-type and mutant p68 kinase-expressing clones. In soft agar, however, clone 12.3, which expresses the mutant kinase, manifested a slight ability to support anchorage-independent growth, whereas no growth was observed for clone Neo or for clone 68.11, which expresses the wild-type p68 kinase (data not shown). No significant difference was observed between the different clones in their response to murine interferon α. This was assessed by interferon dose-dependent induction of the (2′-5′)oligoadenylate synthetase (29) and inhibition of growth in culture (data not shown).

DISCUSSION

Taken together, our results indicate that expression of an inactive human p68 kinase in murine cells results in the reversion of the tumor-suppressing phenotype mediated routinely by low levels of endogenous murine p65 kinase. A functional p68 kinase is required for this tumor-suppressing effect as is the case for the phosphorylation of eIF2 (13, 15). Accordingly, expression of mutant p68 kinase may favor uncontrolled translation as a consequence of repressed endogenous p65 kinase and increased amounts of unphosphorylated functional eIF2 leading to uncontrolled cell proliferation. A correlation between malignant transformation and enhanced translation has been reported by overexpression of the eukaryotic initiation factor eIF4, which binds to the 5′ cap structure of mRNAs (35). However, it seems unlikely that the tumor suppressor function of the p65/p68 kinase is the consequence of eIF2 phosphorylation. Consistent with this, in our NIH 3T3 clones the mutant p68 kinase acts as a dominant-negative inhibitor of the endogenous murine p65 for its tumor suppressor function but not for eIF2 phosphorylation. Functioning of the p65/p68 kinase eIF2 pathway first requires the activation of the kinase (1, 13, 15), in agreement with several earlier reports demonstrating enhanced phosphorylation of the kinase and eIF2 in interferon-treated murine and human cells following viral infection [i.e., in the presence of RNA activators (36–42)]. In clone 68.11, which expresses constitutively the wild-type p68 kinase, eIF2 was found to be phosphorylated after EMC virus infection. In clones Neo or 12.3 (which expresses the mutant kinase), eIF2 phosphorylation was observed only after interferon treatment (to enhance the level of endogenous p65 kinase) and EMC virus infection (15). Under these experimental conditions, eIF2 phosphorylation required the presence of the activator of the transfected or the endogenous protein kinase, but it was not affected by the presence of the mutant p68 kinase. The mutant p68 kinase, therefore, has a differential action on the mechanisms mediating eIF2 phosphorylation or tumor suppression, and in view of this, it is plausible to suggest that this interferon-induced kinase may have two distinct functions.

Previously, we have shown that the p68 kinase is slightly phosphorylated in cells in the absence of virus infection (43), probably in response to some natural activators that exist in cells. Such activators might cause the autophosphorylation of a specific site in the kinase, rendering the kinase functional for the tumor-suppressing phenotype. Because the p65/p68 kinase can be phosphorylated *in vitro* on multiple sites (1, 44),

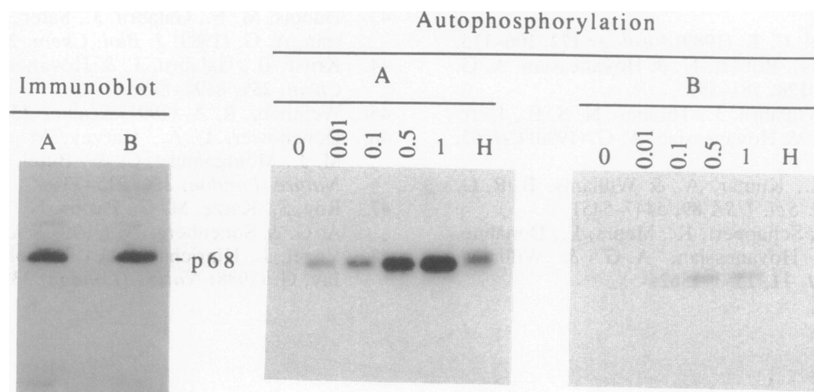

FIG. 4. Inactive protein kinase was recovered from late tumors of mice injected with a clone expressing the wild-type p68 kinase. In one experiment, three out of eight mice injected with clone 68.11, which expresses high levels of the p68 kinase, developed tumors on days 50–54. Cells were recovered from such late tumors on day 65. Analysis of cells from a typical tumor is shown. Extracts corresponding to 10^6 cells (A, clone 68.11; B, 68.11 tumor cells) were analyzed by immunoblotting (*Left*) and by autophosphorylation (*Right*) as described in the legend of Fig. 1. The protein kinase activity (i.e., autophosphorylation) was assayed in the presence of different concentrations of poly(I)·poly(C) (0, 0.01, 0.1, 0.5, and 1 μg/ml) or in the presence of 10 units of heparin per ml (lanes H) as described (8, 15, 21). Autoradiograms are presented.

it may be possible that differential phosphorylation mediates specific conformational modifications conferring the kinase distinct functions. In contrast to other tumor suppressor phosphoproteins, which have nuclear localization, such as p53 and the retinoblastoma gene product (reviewed in ref. 45), the p65/p68 kinase is cytoplasmic and is recovered with the microsomal pellet (1). Interestingly, mutations of p53 are also associated with tumorigenicity (45, 46).

The mechanism by which the p65/p68 kinase mediates a tumor-suppressing phenotype remains to be investigated. For this function, the kinase requires its protein kinase activity, but as yet we have no evidence whether it requires autophosphorylation. The observation that the inactive mutant p68 kinase behaves as a dominant-negative inhibitor of the endogenous p65 kinase suggests that the mutant protein may perhaps sequester a phosphorylatable substrate implicated in the mechanism of tumor suppression. Under physiological conditions, therefore, mutations causing inactivation of p65/p68 kinase or agents inhibiting kinase expression may render normal cells tumorigenic. In this regard, the human immunodeficiency virus *tat* gene product downregulates p68 kinase expression (47), and overexpression of tat in transgenic mice has been associated with development of tumors similar to Kaposi sarcoma (48). Thus it remains possible that suppression of the p68 kinase through its expression or functioning might be one of the pathways inducing malignant cell growth.

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