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The *mdm-2* Oncogene Can Overcome Wild-Type p53 Suppression of Transformed Cell Growth

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Expression of a p53-associated protein, Mdm-2 (murine double minute-2), can inhibit p53-mediated transactivation. In this study, overexpression of the Mdm-2 protein was found to result in the immortalization of primary rat embryo fibroblasts (REFs) and, in conjunction with an activated *ras* gene, in the transformation of REFs. The effect of wild-type p53 on the transforming properties of *mdm-2* was determined by transfecting REFs with *ras*, *mdm-2*, and normal p53 genes. Transfection with *ras* plus *mdm-2* plus wild-type p53 resulted in a 50% reduction in the number of transformed foci (relative to the level for *ras* plus *mdm-2*); however, more than half (9 of 17) of the cell lines derived from these foci expressed low levels of a murine p53 protein with the characteristics of a wild-type p53. These results are in contrast to previous studies which demonstrated that even minimal levels of wild-type p53 are not tolerated in cells transformed by *ras* plus *myc*, E1A, or mutant p53. The *mdm-2* oncogene can overcome the previously demonstrated growth-suppressive properties of p53.

Loss of the growth-suppressive activity of the cellular p53 protein is strongly correlated with the process of transformation. Mutations in p53 are the most common genetic alteration in human tumors (18, 25); in addition, several DNA tumor viruses (simian virus 40 [SV40], adenovirus type 5, and human papillomavirus types 16 and 18) encode transforming proteins that bind to and presumably inactivate (through complex formation or enhanced degradation) wild-type p53 functions (3, 22, 23, 26, 38, 39, 41). The cellular pathways involved in the regulation of p53 function also represent potential targets for alteration during the process of transformation. Recent studies have identified a p53-associated cellular protein, murine double minute-2 (Mdm-2), as a potential regulator of p53 activity (29). The Mdm-2 protein can form oligomeric complexes with wild-type p53 (17, 29), and when *mdm-2* is coexpressed with wild-type p53 in transient expression assays, the ability of p53 to transactivate a p53-responsive test gene is eliminated (29). The fact that *mdm-2* can negatively regulate p53 suggests *mdm-2* could function as an oncogene when overexpressed (in a manner analogous to that of mutant p53 or the SV40 large T antigen). Overexpression of *mdm-2* increased the tumorigenic potential of immortalized NIH 3T3 cells, demonstrating that the Mdm-2 protein does possess oncogenic activity (6).

In this study, the transforming properties of *mdm-2* were further characterized by assaying both the immortalizing and the transforming activities of *mdm-2* in primary rat cells. Transfection of rat embryo fibroblasts (REFs) with *mdm-2* in *cis* with a *neo* gene resulted in an enhancement in the number of drug-resistant colonies, and these colonies were established into immortalized cell lines with a reasonable frequency (30%). Cotransfection of *mdm-2* with an activated *ras* gene resulted in the transformation of REFs. To examine the effect of wild-type p53 overexpression on the oncogenic properties of *mdm-2*, REFs were transfected with *mdm-2* plus *ras* plus wild-type murine p53. Although cotransfection with wild-type p53 resulted in a decrease in the number of transformed foci, analysis of 17 transformed cell lines derived from *ras*-plus-*mdm-2*-plus-wild-type p53 transfections showed that all expressed elevated levels of the Mdm-2 protein and that 9 expressed low levels of a murine p53 with properties characteristic of wild-type p53. None of the cell lines produced p53 with characteristics of a mutant p53 protein. These data suggest that overexpression of the cellular Mdm-2 protein may result in cellular transformation, at least in part, by overcoming the growth-regulatory properties of the p53 protein.

MATERIALS AND METHODS

Cell culture and assays for immortalization and transformation. All cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Primary REFs were prepared from 14- to 15-day-old Fischer 344 whole rat embryos.

To assay immortalization activity, 6×10^5 to 9×10^5 cells were seeded per 10-cm-diameter dish and transfected on day 1 by the calcium phosphate method (12) with 0.5 μg of a control vector containing the *neo* gene (CMV Bam Neo [17]) or CV001, a kind gift from D. George (2), or 1.8 μg of a murine *mdm-2* genomic clone (2A-43/CV001 [6]), also a generous gift from D. George, plus 10 μg of carrier DNA. Expression of the *mdm-2* gene was driven by its own promoter. On day 2, the cells were rinsed and refed with fresh medium; on day 3, the cells were trypsinized and seeded at 5×10^5 or 1×10^6 cells per 15-cm-diameter dish into Geneticin 418 (G418; 600 μg/ml)-containing medium. G418-resistant colonies were cloned into medium without G418. Only clones derived from transfections with *mdm-2* were successfully expanded into 10-cm-diameter dishes. Immortalized cell lines were passaged weekly at split ratios of 1:10 or 1:20 a minimum of five times.

The transforming properties of *mdm-2* were assayed in cooperation with an activated *ras* gene. Primary REFs were seeded into 10-cm-diameter dishes at a density of 3×10^5 to 5×10^5 cells and were transfected on day 1 by the calcium phosphate method (12) with 1.25 μg of an activated *ras* gene (T24 [11]) plus 4.5 μg of a genomic *mdm-2* clone (2A-43/CV001 [6]) plus 10 μg of carrier DNA. On day 2, the cells were rinsed and refed with DMEM supplemented with 10% FBS. The cultures were refed every 5 to 6 days, and the number of transformed foci was determined 2 to 3 weeks

later. Morphologically transformed foci were visible 7 to 10 days following transfection. Transformed foci were cloned and expanded into cell lines. The remaining cells were stained with crystal violet. The effect of wild-type p53 on transformation was determined by transfecting REFs as described above except that 1.25 μg of p53-XA-2, a derivative of a wild-type murine cDNA-genomic (introns 2 and 3) p53 clone (p53-XA [15]), a gift from G. Lozano, was included in the transfection. The p53 gene was expressed under the control of the Harvey murine sarcoma virus long terminal repeat.

Immunoprecipitation and half-life analyses. REFs and transformed cell lines were metabolically labeled for 2 h with [^{35}S]methionine EXPRESS (NEN) at 50 μCi/ml in methionine-free DMEM supplemented with 5% dialyzed FBS. At the end of the labeling period, the cells were washed with phosphate-buffered saline, scraped from the dish, pelleted at 4°C, and stored at −80°C until use. Cell pellets were lysed in lysis buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) for 20 to 30 min on ice with frequent vortexing. Equivalent amounts of trichloroacetic acid (TCA)-insoluble counts were immunoprecipitated as previously described (9). The immunoprecipitated proteins were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels and fluorographed as previously described (9). For half-life determinations, the transformed cells were pulsed for 1 h with [^{35}S]methionine and chased for various periods of time (up to 4 h) in DMEM supplemented with 10% FBS. Equivalent amounts of TCA-precipitable counts from the different time points were immunoprecipitated with PAb246 (42), and the immunoprecipitated proteins were separated on an SDS–7.5% polyacrylamide gel and fluorographed. Labeled p53 protein was solubilized from gel slices with Solvable (DuPont) and counted in a scintillation counter. One half-life was determined to be the time point at which 50% of the zero-time counts remained.

Tumorigenicity. Cell lines established from morphologically transformed foci were assayed for tumorigenicity by subcutaneous injection of 10^6 cells into athymic nude mice. Three different *ras*-plus-*mdm-2*-transformed cell lines were assayed in duplicate. The transformed cells formed palpable tumors within 2 weeks.

RESULTS

Transforming activity of the *mdm-2* gene. To characterize the transforming properties of the *mdm-2* gene, the ability of *mdm-2* to function as an oncogene in conjunction with an activated *ras* gene in primary REFs was first determined. REFs were transfected either with an activated *ras* gene or with an activated *ras* gene plus a genomic murine *mdm-2* gene (2A-43/CV001); 7 to 10 days after transfection, multiple, dense areas of morphologically transformed cells were visible in the dishes that received *ras* plus *mdm-2*. No foci were observed if only the *mdm-2* gene was transfected (7). At 14 days, the number of foci was determined; the results from these studies are presented in Table 1. Cotransfection of *ras* plus *mdm-2* resulted in an average of 28 foci per experiment, demonstrating that *mdm-2* can cooperate with an activated *ras* gene to transform primary cells. The number of foci observed after transfection with *ras* plus *mdm-2* is, on average, less than the number of foci observed after transfection of *ras* plus a murine mutant p53 gene (28 versus 45 [7]) and, on average, 14-fold higher than the number of foci obtained after transfection with *ras* alone (Table 1). Low numbers of transient foci observed with *ras* alone have been previously noted in this assay (17) and are not able to be cloned into established lines (Table 1). To determine whether the *ras*-plus-*mdm-2*-transformed foci represented stably transformed cells, the clonability of the foci was tested. All of the 14 *ras*-plus-*mdm-2*-transformed foci were readily cloned into transformed cell lines composed of highly refractile, rapidly growing cells. Three different *ras*-plus-*mdm-2*-transformed cell lines rapidly formed tumors in nude mice.

TABLE 1. Cooperation of *mdm-2* with activated *ras* to transform primary REFs

Transforming gene(s)	No. of foci					Ratio to *ras* alone	Clonability (%)	Tumorienicity
	Expt 1	Expt 2	Expt 3	Expt 4	Avg			
ras	3	2	3	0	2	1.0	0/5 (0)	NA[a]
ras + *mdm-2*	6	30	35	41	28	14.0	14/14 (100)	3/3

[a] NA, not applicable.

To examine the expression of *mdm-2* in the transformed cell lines, primary REFs and three *ras*-plus-*mdm-2*-transformed cell lines were labeled with [^{35}S]methionine and immunoprecipitated with rabbit preimmune serum PAb421 (a conformation-independent monoclonal antibody specific for rat, mouse, or human p53 [14]) or with a rabbit antiserum that was raised against a bacterially expressed fusion protein of the murine Mdm-2 protein that recognizes both the rat and mouse Mdm-2 proteins (31). The immunoprecipitated proteins were separated on SDS-polyacrylamide gels and subjected to autoradiography. The levels of Mdm-2 in primary REFs are low to nondetectable; however, each of the *ras*-plus-*mdm-2*-transformed cell lines expresses easily detectable levels of the murine Mdm-2 protein (Fig. 1). A complex between Mdm-2 and the endogenous rat p53 was not detected in any of the *ras*-plus-*mdm-2*-transformed cell lines (Fig. 1). Of interest is the observation that the Mdm-2-specific antiserum consistently recognizes additional unidentified cellular proteins (e.g., 60 kDa) in these cell lines (Fig. 1). Whether these are Mdm-2-associated proteins or proteins that share specific epitopes with the Mdm-2 protein is under investigation.

Overexpression of oncogenes capable of cooperating with an activated *ras* gene (e.g., *myc*, E1A, and mutant p53) frequently results in the immortalization of primary rat cells (19–21, 24, 36, 37). The immortalizing activity of *mdm-2* was assayed by transfecting secondary or tertiary REFs with a genomic *mdm-2* gene in *cis* with the *neo* gene (2A-43/CV001) and determining the number of G418-resistant colonies clonable into permanent cell lines. Transfection of *mdm-2* resulted in a significant enhancement of the plating efficiency of these cells (5- to 60-fold over four experiments) over that of REFs transfected with the *neo* gene alone (Table 2). Over 30% (8 of 25) of the colonies that received the *mdm-2* gene were established into cell lines; these cell lines retained the typical fibroblast-like morphology of the parent REFs, and seven of them expressed elevated levels of the *mdm-2* gene (7).

Effect of wild-type p53 on the transformation of REFs by *mdm-2* plus *ras*. Overexpression of wild-type p53 suppresses the development of transformed foci in *ras* cooperation assays using mutant p53, the *myc* gene, or E1A from adenovirus type 5 (5, 8). To assay the effect of wild-type p53 expression on *ras*-plus-*mdm-2* transformation, REFs were

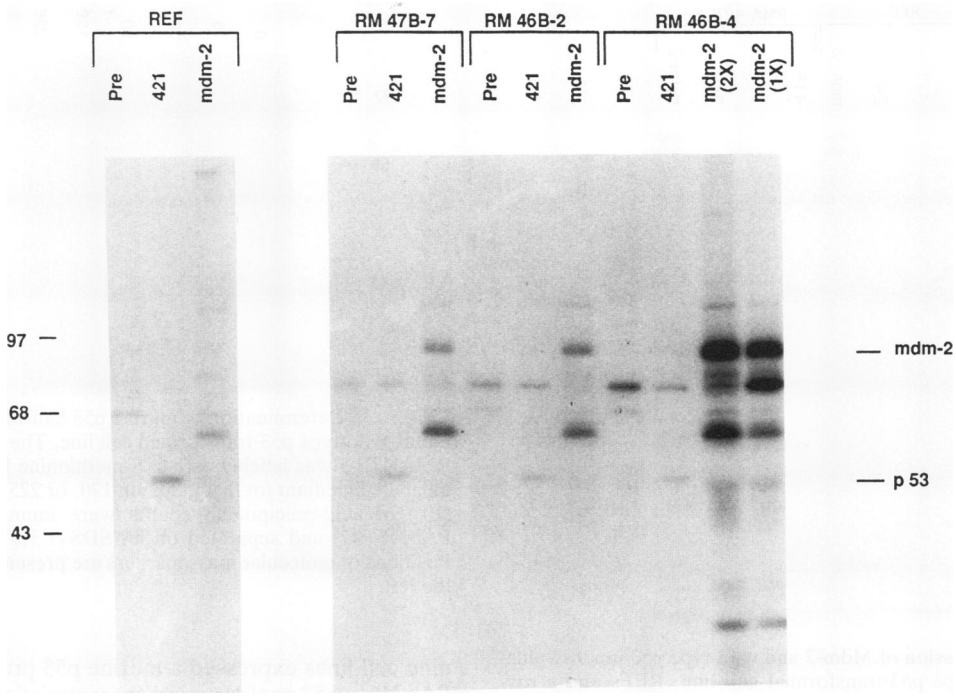

FIG. 1. Immunoprecipitation of Mdm-2 in *ras*-plus-*mdm-2*-transformed cell lines. Approximately 6.5 × 10^6 TCA-precipitable counts were immunoprecipitated from [^{35}S]methionine-labeled extracts of REFs and three *ras*-plus-*mdm-2*-transformed cell lines (RM 47B-7, RM 46B-2, and RM 46B-4, respectively) with rabbit preimmune serum (Pre lanes), PAb421 (a monoclonal antibody specific for p53) (14) (lanes 421), or Mdm-2 antiserum (31) (lanes mdm-2); 2× equals twice the concentration of antibody. The proteins were separated on SDS–7.5% polyacrylamide gels and detected by fluorography. Positions of molecular mass markers are indicated in kilodaltons on the left.

transfected with *ras* plus *mdm-2* plus wild-type murine p53, and the number of foci (relative to those in REFs transfected with *ras* plus *mdm-2* alone) was assayed. There was an approximate 50% reduction of transformed foci in the presence of exogenous wild-type murine p53 (Table 3). This is somewhat lower than the suppression observed previously with use of mutant p53, *myc*, or E1A (65 to 100% reduction) (5, 8). To determine whether elevated levels of p53 could be tolerated in the *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cells, 17 different cell lines derived from three independent *ras*-plus-*mdm-2*-plus-wild-type p53 transfections were labeled with [^{35}S]methionine, and their proteins were immunoprecipitated with PAb416 (14), PAb421, PAb246 (a murine-specific monoclonal antibody that recognizes the wild-type conformation of p53 [10, 28, 42]), or the Mdm-2 antiserum. All of the cell lines expressed elevated levels of *mdm-2*, comparable to those observed in Fig. 1. Most important, 9 of 17 (1 of 6, 2 of 4, and 6 of 7 from the three respective experiments) of these cell lines expressed low levels (comparable to that of the endogenous rat p53) of a murine p53 protein (which migrates slightly faster than the rat p53). The murine p53 was immunoprecipitated with both PAb421 and PAb246. The cell lines expressing murine p53 were composed of highly refractile, rapidly growing cells and were indistinguishable from cell lines transformed by *ras* plus *mdm-2* alone. Representative results of wild-type murine p53 expression in a *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell line are shown in Fig. 2. A band comigrating with *mdm-2* is immunoprecipitated with both PAb421 and PAb246, suggesting the ability of *mdm-2* to bind p53 could be involved in this tolerance of p53 expression. In addition, the affinity of the murine Mdm-2 protein for the murine p53 protein may be higher than that of murine Mdm-2 for rat p53.

Turnover of murine p53 in *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines. Previous studies have demonstrated that both wild-type murine and rat p53s are expressed at low levels in normal cells and that the half-life of the wild-type protein is short, typically 15 to 30 min (32–35). To confirm that the murine p53 expressed in the *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines possesses a short half-life, pulse-chase experiments were conducted with

TABLE 2. Effect of *mdm-2* on the plating efficiency of REFs

Test gene	Colonies/10^6 cells					No. of immortalized clones
	Expt 1	Expt 2	Expt 3	Expt 4	Avg (no. of trials)	
CMV Bam Neo	5	ND[a]	ND	ND	5 (1)	
CV001	ND	8	42	6.5	19 (3)	0/8
mdm-2	317	144	235	100	199 (4)	8/25

[a] ND, not determined.

TABLE 3. Effect of wild-type p53 on the transformation of REFs by *ras* plus *mdm-2*

Test genes	No. of foci					Ratio to *ras* + *mdm-2*
	Expt 1	Expt 2	Expt 3	Expt 4	Avg	
ras + wild-type p53	0	1	0	0	0	0
ras + *mdm-2*	30	41	56	25	38	1.0
ras + *mdm-2* + wild-type p53	15	31	21	15	20	0.52

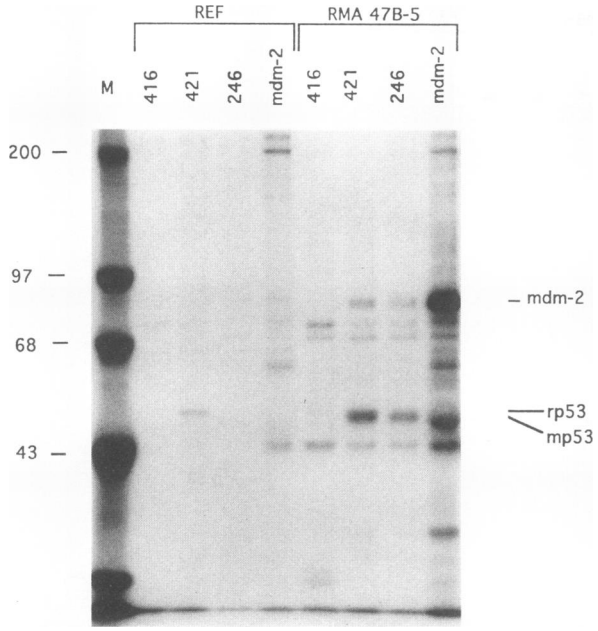

FIG. 2. Coexpression of Mdm-2 and wild-type p53 in a *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell line. REFs and a *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell line (RMA 47B-5) were labeled with [^{35}S]methionine and immunoprecipitated with PAb416, a control monoclonal antibody that recognizes the SV40 large T antigen (14) (lanes 416), PAb421 (lanes 421), PAb246, a conformation-dependent, murine p53-specific monoclonal antibody (42) (lanes 246), or anti-Mdm-2 antiserum (lanes mdm-2) (31). Approximately 4.0×10^6 and 1.2×10^7 TCA-precipitable counts were immunoprecipitated from labeled extracts of REFs and RMA 47B-5 cells, respectively. The proteins were separated on an SDS–7.5% polyacrylamide gel and fluorographed. Relative positions of the molecular size standards are shown on the left in kilodaltons. Positions of the migration of rat p53 (rp53), murine p53 (mp53), and Mdm-2 are indicated on the right.

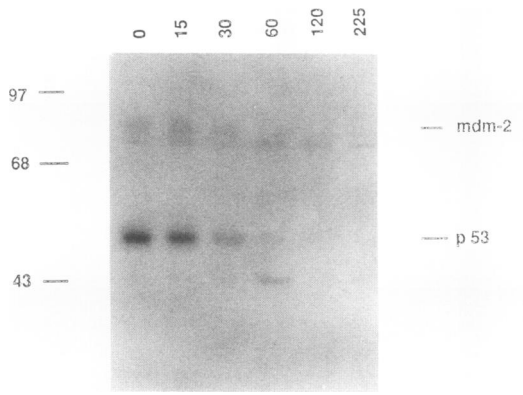

FIG. 3. Determination of murine p53 half-life in a *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell line. The transformed cell line RMA 47B-5 was labeled with [^{35}S]methionine for 1 h and chased in unlabeled medium for 0, 15, 30, 60, 120, or 225 min. Equal amounts (10^7) of acid-precipitable counts were immunoprecipitated with PAb246 (42) and separated on an SDS–7.5% polyacrylamide gel. Positions of molecular mass markers are presented in kilodaltons on the left.

three independent *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines. In each of the three cell lines, the half-life of the PAb246-reactive p53 was short (15 to 25 min). Similar results were obtained when PAb421 was used (7). The half-life of Mdm-2 was also determined in two of the *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines and was found to be short, approximately 30 min (7). Representative results are presented in Fig. 3 and summarized in Table 4.

The murine p53 in *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines has wild-type properties. The murine p53 expressed in the *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines has many properties indicative of a wild-type p53 protein (Table 4). First, the levels of murine p53 are very low in all of the nine cell lines examined, comparable to the levels of the endogenous rat p53 in REFs. Consistent with the low levels of murine p53 expression, the half-life of the murine p53 in three different cell lines was found to be short (15 to 25 min). Previous studies have demonstrated that mutant p53 proteins are typically expressed at elevated levels in transformed cell lines (4, 9, 17), and this is primarily the result of an increased stability of the mutant proteins (9, 13, 17). Second, no association of the murine p53 protein with the cellular heat shock protein hsc70 was detected. The ability to bind to hsc70 has been associated with mutant p53 proteins (4, 9, 40). Third, each of the nine cell lines expressed a murine p53 protein reactive with PAb246, a p53 conformation characteristic of a wild-type p53 protein (10, 28). In addition, the proteins of three of these cell lines were analyzed by immunoprecipitation with PAb240, a monoclonal antibody that recognizes some mutant p53 proteins (1, 10), and none of the cell lines expressed p53 that could bind PAb240 (7). That the exogenous p53 shares these characteristics strongly argues that the transfected murine p53 genes are present in the wild-type form.

DISCUSSION

In this study, the growth-promoting properties of the Mdm-2 protein have been investigated in primary rat cells. The results presented demonstrate that, like *myc*, E1A, or mutant p53, the *mdm-2* gene can immortalize primary REFs and can cooperate with an activated *ras* gene to transform primary REFs. Although these oncogenes (*mdm-2*, *myc*, E1A, and mutant p53) have similar transforming properties in vitro and can be classified as oncogenes capable of cooperating with *ras*, the mechanisms of action are not yet understood. Overexpression of these oncogenes may alter the same or different cellular pathways. The *mdm-2* gene encodes a protein with characteristics of a transactivator (an acidic domain and a nuclear localization signal [6]) and therefore may enhance cell growth by directly altering gene expression. Although *mdm-2* may transform by activating or repressing cellular pathways that are independent of p53 control, the fact that *mdm-2* expression can abrogate the ability of p53 to transactivate a test gene (29) supports a model of transformation involving loss of p53 function.

TABLE 4. Characteristics of murine p53 in *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cell lines

Cell line	Monoclonal antibody binding			hsc70 binding	Half-life (min)
	PAb421	PAb246	PAb240		
RMA 47B-5	+	+	–	–	25
RMA 61B-2	+	+	–	–	15
RMA 61B-5	+	+	–	–	15

Transformation of primary rat cells by mutant p53 and *ras* is also thought to involve the loss of the endogenous rat wild-type p53 function (dominant loss of function) (25). Thus, *mdm-2* and mutant p53 may transform by utilizing at least one common mechanism.

The tolerance to wild-type p53 expression in *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cells provides additional evidence that *mdm-2* expression can overcome the negative effects of p53 on cell proliferation. This result stands in contrast to that observed upon cotransfection with *ras*, E1A, and wild-type p53 genes; in that case, cell lines expressing murine p53 contain only obviously mutant forms of the p53 protein (8). The fact that the number of *ras*-plus-*mdm-2* foci is consistently reduced following transfection with wild-type p53, coupled with the consistently low levels of wild-type p53 expression, suggests there is a selection against high levels of the wild-type protein in the transformed cell clones. Thus, although there is a tolerance to p53 expression in the presence of high levels of Mdm-2, this is limited to only low levels of the p53 protein.

The tolerance of wild-type murine p53 expression in *ras*-plus-*mdm-2*-plus-wild-type p53-transformed cells is a phenotype unique to this cellular oncogene; expression of exogenous p53 in primary rat cells has been previously noted only with use of the SV40 large T antigen gene as a cooperating oncogene (27). Of interest is the observation that p53 binding is not necessary for this response; cell lines expressing an SV40 large T antigen mutant deficient for p53 binding also tolerated exogenous wild-type p53 expression (27). Thus, there are apparently functions provided by the large T antigen (in addition to complexing p53) that are involved in the inactivation of p53-regulated pathways. By analogy, the Mdm-2 protein may also possess growth-promoting functions that can overcome the regulation of cell growth by p53 in the absence of complex formation.

Whether complex formation between Mdm-2 and p53 is necessary for part or all of the transforming activity of the Mdm-2 protein is unclear. A complex between *mdm-2* and p53 is not detectable in the *ras*-plus-*mdm-2*-transformed cell lines, and immunoclearing experiments with Mdm-2 antiserum have confirmed that there is free p53 present in asynchronously growing cultures of these transformed cells (7). Thus, if complex formation is critical for the inactivation of p53, only a small subset of the p53 molecules in the cell are present in an active form. Alternatively, the complex may transiently form only at distinct control point(s) in the cell cycle. It is also possible that the ratio of *mdm-2* to p53 is important for transformation; overexpression of *mdm-2* may directly promote cell growth by raising the level of free, presumably active, *mdm-2*. Experiments are in progress to distinguish among these possibilities.

The results presented in this report form the basis for a prediction about the inactivation of p53 in human tumors. If *mdm-2* can act as an oncogene, there should exist tumors that have amplified the *mdm-2* gene. Furthermore, if the transforming activity of *mdm-2* results, at least in part, from the ability of *mdm-2* to abrogate p53 activity, these tumors should also express wild-type p53. Recent studies from the Vogelstein laboratory have demonstrated the amplification of the *mdm-2* gene in liposarcomas, malignant fibrous histiocytomas, and osteosarcomas, and five tumors with elevated *mdm-2* levels possessed wild-type p53 (30). Thus, the *mdm-2* gene product is an oncogene that may act, at least in part, through the inactivation of the p53 tumor suppressor gene product. Experiments designed to determine the mechanism(s) by which *mdm-2* acts to overcome the regulation of cell growth by p53 are in progress.

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