mdm2 expression is induced by wild type p53 activity

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We have recently characterized a 95 kDa protein, p95, which exhibits enhanced binding to temperature-sensitive p53 (ts-p53) when cells are shifted down to 32.5°C, a temperature at which ts-p53 possesses wild-type (wt)-like activities. In the present study we show that p95 is a product of the mdm2 putative proto-oncogene. The enhanced complex formation of mdm2 with ts-p53 in cells maintained at 32.5°C is due to an elevation in total mdm2 protein levels following the temperature shift. We further demonstrate that the induction of mdm2 expression by wt p53 activity is at the mRNA level. The induction occurs with very rapid kinetics and does not require de novo protein synthesis, suggesting a direct involvement of p53 in the process. Based on these data and on recent findings implicating p53 as a transcription factor, we suggest that the mdm2 gene is a target for activation by wt p53. In view of the ability of mdm2 to act as a specific antagonist of p53 activity, this induction process may serve to tightly autoregulate p53 activity in living cells.

Key words: growth control/mdm2/p53/tumor suppressor

Introduction

The p53 tumor suppressor gene is frequently inactivated in many human malignancies (reviewed by Hollstein et al., 1991; Oren, 1991; Tominaga et al., 1992). Tumor derived as well as in vitro transformed cells often lack functional wild type (wt) p53. When the expression of wt p53 is induced in such cells, the outcome is often growth arrest, implying that p53 may play a role in the regulation of cell proliferation (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Michalovitz et al., 1990). The activation of wt p53 was also shown to promote differentiation and active cell death in certain cellular contexts (Shaulsky et al., 1991; Yonish-Rouach et al., 1991; Feinstein et al., 1992; Shaw et al., 1992). Recently, it has been suggested that p53 may play a key role in preventing genomic instability, presumably through the orchestration of a G1 growth arrest upon exposure to DNA damaging agents (Kastan et al., 1991; Kuwertz et al., 1992; Lane, 1992). These activities are all likely to account, in one way or another, for the ability of wt p53 to act as a tumor suppressor.

p53 possesses features of a transcription factor (reviewed by Levine et al., 1991; Montenarh, 1992; Oren, 1992; Vogelstein and Kinzler, 1992). This includes the presence of a potent transactivation domain in the N-terminal part of the protein (Raycroft et al., 1990, 1991; Fields and Jang, 1990; O'Rourke et al., 1990; Shohat-Foord et al., 1991; Unger et al., 1992), and an ability to engage in sequence-specific DNA binding (Bargonetti et al., 1991; Kern et al., 1991; El-Deiry et al., 1992; Funk et al., 1992). In fact, p53 has now been shown to be capable of directly activating the transcription of promoters containing a p53 binding site (Farmer et al., 1992; Funk et al., 1992; Kern et al., 1992; Zambetti et al., 1992). So far, however, it has been difficult to identify genes which serve as biologically significant targets for activation by wt p53. One potential target is the muscle-type creatine kinase gene (MCK; Weintraub et al., 1991), whose activation involves sequence-specific binding by p53 (Zambetti et al., 1992). However, the relevance of MCK to the known activities of p53 is unclear.

In addition to its ability to activate transcription from promoters containing a cognate binding site, wt p53 can also repress the activity of a variety of promoters, some of which correspond to genes whose expression is positively correlated with cell proliferation or increased malignancy (Ginsberg et al., 1991a; Mercer et al., 1991; Santhanam et al., 1991; Chin et al., 1992). However, there is no evidence for the direct binding of p53 to sequence elements within or next to the pertinent genes. It is of note that a number of DNA tumor virus proteins are capable of interfering with the transcriptional effects of p53 (Farmer et al., 1992; Lechner et al., 1992; Yew and Berk, 1992). The identification of relevant target genes, which are subject to regulation by wt p53, is likely to shed light on the molecular basis for its tumor suppressor activity.

An alternative approach towards understanding the biochemical functions of p53 involves the search for cellular p53 binding proteins. Such studies have recently led to the finding that p53 is capable of forming specific complexes with the product of the mdm2 gene (Momand et al., 1992). This gene was first identified and cloned on the basis of its amplification in a highly tumorigenic derivative of NIH-3T3 cells, and has subsequently been shown to confer tumorigenic properties upon transfected cells (Fakharzadeh et al., 1991). The product of the mouse mdm2 gene is a protein with a predicted molecular mass of 54 kDa. Even though its precise biochemical nature still remains to be determined, its predicted amino acid sequence indicates that it possesses a putative nuclear localization signal as well as two zinc fingers, suggesting that it may be a DNA binding protein (Fakharzadeh et al., 1991). The mdm2 protein can associate with both mutant and wt p53 (Hinds et al., 1990; Momand et al., 1992). Furthermore, excess mdm2 can abrogate transcriptional activation by transfected wt p53 (Momand et al., 1992). Hence, overexpressed mdm2 may serve as a negative regulator of p53 function. The significance of this observation is supported by the identification of human sarcomas exhibiting amplification of the mdm2 gene (Oliner et al., 1992). This presumably abrogates the function of p53 in the tumor cells, bypassing the need for structural alterations in their p53 genes (Vogelstein and Kinzler, 1992).
We have recently reported the presence of tight complexes between p53 and a specific cellular protein in cells induced to undergo p53-mediated growth arrest (Barak and Oren, 1992). In rodent fibroblasts carrying the temperature-sensitive (ts) p53 mutant p53val135, a temperature downshift to 32.5°C results in the induction of wt-like p53 activities (Michalovitz et al., 1990). This activation, which is also correlated with the translocation of the protein from the cytoplasm into the nucleus (Gannon and Lane, 1991; Ginsberg et al., 1991b; Martinez et al., 1991), eventually leads to a reversible growth arrest. In these cells growth arrested at 32.5°C, some of the p53 is associated with a polypeptide of an apparent molecular weight of 95 kDa (p95; Barak and Oren, 1992). While ample quantities of p53—p95 complexes are found at 32.5°C, there are hardly any such complexes at 37.5°C, when cells proliferate continuously and express predominantly the mutant form of the ts p53 (Barak and Oren, 1992). These facts suggested a possible relationship between p53-mediated growth arrest and the formation of p53—p95 complexes.

We now report that p95 is in fact the product of the mdm2 gene. Furthermore, we demonstrate that the pronounced accumulation of p53—p95 complexes at 32.5°C is due to the enhanced synthesis of the mdm2 polypeptide; this reflects a rapid increase in mdm2 mRNA levels following the induction of wt p53 activity. The induction does not require any de novo protein synthesis, supporting a direct effect of p53. Our findings suggest that the mdm2 gene is a relevant target for direct positive regulation by p53.

Results

p95 is identical to mdm2

We have recently characterized a 95 kDa polypeptide (p95), whose binding to temperature-sensitive (ts) p53 is increased in cells upon shift to 32.5°C (Barak and Oren, 1992). p95 appears to share several characteristics with the recently described p53 binding mdm2 protein (Momand et al., 1992). First, both proteins migrate to approximately similar positions on SDS—PAGE (Hinds et al., 1990; Barak and Oren, 1992; Momand et al., 1992). Second, both proteins are heavily phosphorylated (Barak and Oren, 1992; Momand et al., 1992). Third, p95 is a highly acidic protein, as determined by 2D protein electrophoresis (Y.Barak, unpublished results); likewise, the deduced amino acid content of mdm2 (Fakhrazadeh et al., 1991) predicts that its pl should be very low. Fourth, the half-life of both proteins in their p53-bound form is very short (Hinds et al., 1990; Y.Barak, unpublished results).

To determine unequivocally whether p95 and mdm2 are identical, we isolated four mdm2 cDNA clones from a normal mouse testis cDNA library (see Materials and methods). DNA sequencing confirmed the identity of the clones, and revealed one alternatively spliced variant closely similar to a previously described form (data not shown). We subsequently constructed a pGEX2T—mdm2 fusion plasmid harboring the C-terminal 431 amino acids of mdm2, and expressed it in Escherichia coli. The resultant fusion protein was purified from the urea-insoluble inclusion bodies by SDS—PAGE, and injected into rabbits (see Materials and methods). Polyclonal sera were assayed for reactivity with in vitro translated mdm2.

For the studies described below we chose to use Clone 6 (C6) cells, derivatives of rat embryo fibroblasts (REF) transformed by Ha-ras and the temperature-sensitive (ts) mutant p53val135 (Pinhasi-Kimhi et al., 1986). In these cells large amounts of p95 are complexed with p53 at 32.5°C (Barak and Oren, 1992). The anti-GST—mdm2 serum (α-mdm2) was employed in an immunoprecipitation reaction with C6 extracts. The immune complexes were Western blotted and reacted with the anti-p53 monoclonal antibody (mAb) PAb248. Figure 1A shows that p53 could be detected in α-mdm2 precipitates of C6 extracts, provided that the cells were kept at 32.5°C (lane 2). No p53 could be detected in α-mdm2 precipitates of C6 maintained continuously at 37.5°C (lane 4), although the extracts of these cells and the former contained comparable amounts of p53 (lanes 5 and 6). As expected, no p53 precipitated with pre-immune serum from extracts of cells maintained at either temperature (Figure 1A, lanes 1 and 3). In a reciprocal experiment the same extracts were first immunoprecipitated with the p53-specific monoclonal antibody PAb421, and the blot was probed with α-mdm2 serum (Figure 1B). The mdm2 polypeptide was evident when the cells had been maintained at 32.5°C (Figure 1B, lane 2), but not at 37.5°C (lane 4), similar to the co-precipitation pattern of p95 with p53 (Barak and Oren, 1992). The SV40 large T antigen-specific mAb PAB419, used as a control, did not precipitate any mdm2 cross-reactive material (lanes 1 and 3). The series of bands referred to as CR95 (Figure 1A, lanes 2, 5 and 6) represent as yet uncharacterized polypeptides, encoded by the mouse p53 gene transfigured into C6 (Y.Barak, unpublished results). Apparently, the mdm2 binding potential of CR95 is comparable with that of p53 itself. These data indicate that mdm2 and p95 are indistinguishable with respect to their ability to co-precipitate with ts-p53.

It is still possible that p95 and mdm2 could be distinct polypeptides which exhibit similar p53 binding patterns and migrate fortuitously to the same position on SDS—PAGE. To rule out this possibility sequential immunoprecipitation was performed. A 35S-labeled extract of C6 cells maintained at 32.5°C for 24 h was subjected to three consecutive immunoprecipitations with α-mdm2 (Figure 1C, lanes 1–3) and the mdm2-cleared extract was then immunoprecipitated with PAb421 (lane 4). The results clearly demonstrate that α-mdm2 antibodies, while removing only a minor fraction (~5%) of p53 (compare lanes 1–3 with lane 5), depleted the extracts of almost all their p53-bound p95 (compare lanes 4 and 5). This argues very strongly that the 95 kDa polypeptides co-precipitating with p95 (p95) are equivalent to mdm2. It is notable that only a small fraction of p53 in these cells is in complex with mdm2, even at 32.5°C.

mdm2 protein levels are increased in C6 cells at 32.5°C

The enhanced binding of mdm2 to p53val135 in cells maintained at 32.5°C could be due to a selective interaction of mdm2 with wild-type (wt) rather than mutant p53. However, we have previously demonstrated that the wt and mutant conformations of p53val135 were equally potent in binding mdm2 (Barak and Oren, 1992). Therefore, the increased amounts of co-precipitating mdm2 must result either from a change in the ability of this protein to bind p53 or from the production of elevated levels of the mdm2 protein in cells exposed to wt p53 activity.

Therefore, we used α-mdm2 antibodies to assess the mdm2 status in C6 before and after the temperature shift.
C6 extracts were subjected to SDS–PAGE either directly or following immunoprecipitation with α-mdm2 serum. Following blotting and reaction with the same serum it became clear that the overall amounts of mdm2 protein in cells maintained at 37.5°C are much lower than at 32.5°C (Figure 2A and B). This result was corroborated by an immunoprecipitation experiment with extracts of 35S-labeled C6 cells maintained at either temperature (Figure 2C). α-mdm2 serum precipitated ample quantities of mdm2 from cells maintained at 32.5°C, as well as a significant amount of bound p53. On the other hand, hardly any mdm2 could be brought down from cells maintained at 37.5°C.

Cell staining experiments further extended the above observations. Consistent with the presence of a putative nuclear localization signal in the deduced amino acid sequence of mdm2 (Fakharzadeh et al., 1991), the protein is indeed nuclear. Strong nuclear staining with α-mdm2 antibodies was observed in cells maintained at 32.5°C (Figure 3A and C). At 37.5°C, however, only a weak, diffuse staining pattern was observed (Figure 3B and D),

Fig. 1. p95 is a product of the mdm2 gene. (A and B) Clone 6 cells were either grown continuously at 37.5°C or shifted to 32.5°C for 24 h, as indicated above each lane. Aliquots of extracts derived from similar numbers of cells were applied on to 9% SDS–PAGE either directly (A, lanes 5 and 6) or following immunoprecipitation with either pre-immune serum (PI), α-mdm2 serum, the p53-specific mAb PAb421 (421) or the anti-SV40 large T antigen mAb PAb419 (419), as indicated above each lane. The gels were blotted on to PVDF membranes (Bio-Rad), reacted with either the p53-specific mAb PAb248 (A) or α-mdm2 serum (B), and then with either goat anti-mouse or protein A, respectively, coupled to horseradish peroxidase (HRP). The blots were developed using the ECL detection kit (Amersham). Positions of p53, CR95 (see text) and mdm2 are indicated. (C) Clone 6 cells maintained at 32.5°C for 24 h were labeled with [35S]methionine. A cell extract aliquot containing 4 × 10⁶ acid-insoluble c.p.m. was immunoprecipitated with α-mdm2 serum (lane 1). The supernatant from this immunoprecipitation was re-precipitated twice consecutively with the same serum (lanes 2 and 3) and then with PAb421 (lane 4). A similar cell extract aliquot was immunoprecipitated directly with PAb421 as a control (lane 5). Positions of molecular size markers (M), p53, hsc70 and p95/mdm2 are indicated.
which was only slightly more intense than that seen upon staining of the cells with pre-immune serum (Figure 3E). This staining was similar to that obtained with anti-GST serum (Figure 3F). All the above observations confirm that the enhanced binding of mdm2 to p53val135 at 32.5°C is a direct outcome of elevated mdm2 protein levels at this temperature.

Two additional polypeptides, p80 and pp58, exhibit a p53 binding pattern similar to that of p95 (Barak and Oren, 1992). The Western blots shown in Figures 1B and 2 detect an mdm2-related antigen of ~58 kDa, which could be identical to pp58. Furthermore, pulse–chase analysis of C6 cells maintained at 32.5°C revealed gradual accumulation and subsequent disappearance of an mdm2 co-precipitating 58 kDa polypeptide during the early chase period (data not shown), suggesting that it may represent a relatively stable intermediate in the course of mdm2 degradation. In additional blots other cross-reactive bands in the range of 58–85 kDa were sometimes detected (data not shown), suggesting that they are also mdm2-derived polypeptides, possibly including p80 as well.

**Elevated mdm2 mRNA levels follow the induction of wt p53 activity**

Conceivably, the increase in mdm2 protein levels at 32.5°C could be the result of elevated mRNA levels. To examine this possibility, we performed a Northern blot analysis of cytoplasmic RNA prepared from C6 cells maintained at either 37.5°C or 32.5°C for 24 h. As seen in Figure 4A an mdm2-specific probe hybridized to a major ~3.3 kb transcript, as well as to a minor ~1.8 kb one. Both types of transcripts were present in C6 maintained at 32.5°C at much higher levels than in the same cells maintained at 37.5°C (compare lanes 1 and 2). The levels of GAPDH mRNA, probed as a control, were similar in both RNA samples (Figure 4B, lanes 1 and 2). A similar temperature down-shift could not elicit an elevation in mdm2 mRNA in three rat embryo fibroblast (REF) derived cell lines, which do not express the ts-p53; these include clone 51, co-transformed by ras and myc (Michalovitz et al., 1990), and expressing small amounts of endogenous, supposedly wt, p53 (M.Oren, unpublished observations; Figure 4A, lanes 3 and 4); R-Phe132 #5, co-transformed by Ha-ras and the non-conditional p53 mutant, p53phe132 (Michalovitz et al., 1990; lanes 5 and 6); R-Cys270 #2, co-transformed by H ara s and p53cys270 (Haley et al., 1990; data not shown). Thus, overexpression of wt p53 activity specifically leads to an increase in mdm2 mRNA.

To gain better insight into the relationship between p53 activation and mdm2 induction, we determined the kinetics of the process following the temperature down-shift. As seen in Figure 5, mdm2 mRNA started accumulating rather rapidly, and a significant augmentation was evident as early as 2 h following the temperature shift (Figure 5A, lane 2). mdm2 mRNA further accumulated at later time points (lanes 3 and 4). Unlike mdm2, many other genes are down-regulated by the introduction of wt p53 activity into cells (Ginsberg et al., 1991a; Mercer et al., 1991; Santhanam et al., 1991; Chin et al., 1992; Lechner et al., 1992). To compare the kinetics of these two opposing processes, the
same blot was re-hybridized with probes specific for myc and PCNA. myc mRNA levels decline quickly (Figure 5C), reaching a minimum within 2 h following the shift to 32.5°C (lane 2). The levels of PCNA transcripts, on the other hand, declined with much slower kinetics (Figure 5D); this could either reflect a differential response of these two genes to wt p53, or simply a relative difference in the stabilities of their transcripts. These results confirm previous findings, and demonstrate that various growth-related genes respond differentially to the overexpression of wt p53 activity.

Next, we checked whether de novo protein synthesis was required in order to mediate between the temperature shift and the increase in mdm2 mRNA. To this end the translation inhibitor cycloheximide (CHX) was applied to C6 cultures 1 h before the temperature down-shift followed by an additional 4 h at 32.5°C in the presence of CHX. RNA was extracted from these cells, as well as from cells maintained for 4 h at 32.5°C in the absence of the drug. The results are shown in Figure 6. The addition of CHX had a positive effect on mdm2 mRNA levels, even at 37.5°C. This was seen in C6 cells (Figure 6A, lanes 3 and 4), as well as in the cell lines R-Phe132 #5 (lanes 5 and 6) and R-Cys270 #2 (data not shown), both of which express non-ts p53 mutants coupled with very low basal levels of mdm2 mRNA. These data suggest that a short-lived protein may be required either for transcriptional repression of the mdm2 gene or for rapid degradation of the corresponding transcript. Most importantly, when C6 cells were shifted to 32.5°C, the dramatic increase in mdm2 mRNA levels was not prevented by the presence of CHX (compare lanes 2 and 4). In fact, the actual levels of the transcript reflected the combined positive effects of wt p53 activation and protein synthesis inhibition. Hence, the induction of mdm2 by wt p53 does not depend on de novo protein synthesis. This strongly suggests that the p53 protein itself is responsible for the increase in mdm2 levels.

**Discussion**

In this study we present data showing that the expression of the mdm2 gene product, a p53 binding protein, is positively regulated by the transient induction of wt p53 activity. To the best of our knowledge, this is the first demonstration of the ability of p53 to upregulate the expression of a gene whose relevance to p53 is clearly evident. Previously, wt p53 has been shown to induce the expression of the MCK gene through the specific interaction with cognate DNA elements located ~3 kb upstream of the transcriptional start site (Weintraub et al., 1991; Zambetti et al., 1992). It is not immediately obvious how the induction of MCK expression relates to the reported biological activities of wt p53. In this sense, the relationship between p53 function and the expression of mdm2 is much better established.

As wt p53 induces a growth arrest in C6 cells, one could argue that the induction of mdm2 expression is a consequence of the growth arrest rather than being directly attributable to p53 itself. This possibility, however, seems unlikely for
a number of reasons. First, the kinetics of mdm2 transcript accumulation in response to wt p53 activation is very rapid, and a pronounced effect is seen already within 2 h (Figure 5). In contrast, the growth arrest in C6 is observable only 6–12 h after the shift down to 32.5°C (Gannon and Lane, 1991). Second, the process is independent of de novo protein synthesis (Figure 6), suggesting that mdm2 gene expression is directly modulated by p53. Finally, wt p53 activation at 32.5°C also induces mdm2 expression, with similar kinetics, in myeloid leukemic cells transfected with the ts p53 (N. Levi, E. Yonish-Rouach, M. Oren, and A. Kimchi, in preparation). In these cells, wt p53 activity elicits apoptosis (Yonish-Rouach et al., 1991) without exerting any measurable growth inhibitory effect (Yonish-Rouach et al., 1993). For the time being, it still remains to be established whether the induction of mdm2 by wt p53 occurs at the transcriptional or posttranscriptional level. Furthermore, as the mdm2 gene promoter has not been defined, it is still unknown whether the effect of p53 on mdm2 expression involves interactions with specific DNA targets.

The mdm2 gene was first identified on the basis of its putative oncogenic activity (Fakharzadeh et al., 1991). Moreover, it has been implicated as a negative regulator of p53, whose overexpression may mimic the effects brought about by mutational inactivation of the p53 gene (Momand et al., 1992; Oliner et al., 1992). At first glance, it would seem paradoxical that this gene would be a target for positive regulation by the growth-inhibitory form of p53. Yet, this may not be so surprising, since the activity of p53 in a normal cell is by and large regulated at the level of the protein rather than of the gene. This probably has to do with the relatively long half-life of the p53 mRNA (Coulier et al., 1985; Dony et al., 1985), making transcriptional regulation a rather ineffective option. On the other hand, the very short half-life of the protein in normal cells (Oren et al., 1981; Rogel et al., 1985) indicates that there is indeed a need to control cellular p53 activity levels very tightly and precisely. The notion that p53 activity is controlled at the protein level is also supported by other observations, such as the stabilization of p53 following exposure to DNA-damaging agents (Maltzman and Czyzyc, 1984; Kastan et al., 1991) and the direct targeting of the p53 polypeptide by a variety of viral oncoproteins (Levine et al., 1991). If p53 is normally activated as part of a signal transduction pathway, one potential way to maintain tight control of this process could be through the functional inactivation of p53 once it has delivered the signal. This goal could be achieved through the activation of a gene whose product can, in turn, render the p53 protein inactive. The product of the mdm2 gene may mediate such a switch-off mechanism. Once sufficient amounts of p53-induced transcripts have been produced, the accumulated mdm2 protein will lead eventually to the termination of the p53-mediated signal. However, when p53 is overexpressed at very high levels, such as in C6, this feedback mechanism fails to operate due to the large excess of p53 over mdm2.

Finally, even though excess mdm2 can negate some of the activities of wt p53 in experimental systems based on p53 overexpression (Momand et al., 1992), this may not necessarily be the only way in which mdm2 affects the
function of p53. The predicted structure of the mdm2 protein suggests that it may be capable of sequence-specific DNA binding (Fakhrazadeh et al., 1991). One attractive possibility is that p53—mdm2 complexes may possess a novel DNA binding specificity, different from that of p53 alone. This may serve to regulate a specific and distinct set of genes, as a second step in the propagation of p53-mediated signals. Irrespective of whether the induction of mdm2 expression by p53 is part of a negative feed-back loop or a positive component in p53-mediated effects, our observations now provide interesting new clues towards a better understanding of the mode of action of p53.

Materials and methods

Cloning of mdm2 cDNA

A normal mouse testis cDNA library (Fischman et al., 1990) was screened with the 20mer GCTCCCGAGTTGACTCATCT, spanning nucleotides 727–708 of the published murine mdm2 sequence (Fakhrazadeh et al., 1991). The identity of four mdm2 cDNA clones obtained in the initial screen was further confirmed by cross-hybridization to a PCR-derived mdm2 cDNA probe stretching from nucleotide 216 to 600 of the published sequence, and by DNA sequencing. Two of these clones spanned the entire mdm2 coding region, including one previously reported splicing variant, and their 5' portions extended to a position which was proximal to the reported terminus of mdm2 mRNA.

Preparation of α-mdm2 antibodies

The mdm2 sequence corresponding to amino acids 59–489 of the coding region was fused in-frame to PGEX-2T. Expression of the chimera was induced in E. coli, strain HB101, by IPTG, and the bacteria were lysed using lysozyme and detergent (Sambrook et al., 1989). The glutathione-S-transferase–mdm2 (GST–mdm2) chimeric protein was confined to inclusion bodies and was resistant to extraction with 8 M urea. The urea-insoluble material was boiled in Laemmli sample buffer for 30 min and resolved by 7.5% SDS–PAGE. Slices of the gel, each containing 50 µg fusion protein purified by this method, were mixed with Freund’s adjuvant and injected into NZW rabbits at intervals of 2–3 weeks. The first injection was in the presence of complete adjuvant, while incomplete adjuvant was used for further boosts. Rabbits were bled once before the course of injections to yield pre-immune serum, and 10–14 days following each boost, starting from the third boost. Actual titers of the polyclonal sera were examined by their ability to immunoprecipitate in vitro translated mdm2.

Cell lines, antibodies and probes

Clone 6, R-Phe132/5 #5 and R-Cys270 #2 are rat embryo fibroblast (REF) lines generated by co-transformation with Ha-ras and p53, respectively. The monoclonal antibodies (mAbs) used in this study are anti-p53 mAbs PAB421 (Harlow et al., 1981) and PAB248 (Yewdell et al., 1986), and the anti-SV40 large T antigen mAb PAB419 (Harlow et al., 1981). The generation of pre-immune and anti-mdm2 rabbit sera is described above. Hybridoma supernatants containing mdm2 antibodies were diluted 1:10 for immunoprecipitations and 1:40 for Western blots, while rabbit sera were diluted 1:500 and 1:250, respectively. Anti-GST rabbit serum was a gift from D. Michael.

Probes used for Northern blot hybridization include an mdm2-specific probe spanning from nucleotide 3 to 600 of the published sequence (Fakhrazadeh et al., 1991), derived from a full-length cDNA clone; a murine GAPDH cDNA specific 1.3 kb probe; a 2.9 kb fragment from murine mva genomic DNA (Bernard et al., 1983); and a 0.9 kb murine PCNA cDNA probe (Almendral et al., 1987).

Immunoprecipitations, Western blots and immunofluorescent staining

Immunoprecipitation was performed as described (Maltzman et al., 1981). Immunoprecipitated proteins were resolved by 9% SDS–PAGE. Gels containing radiolabeled material were fixed and fluorographed in 1 M sodium salicylate. For Western blots gels were transferred on to PVDF membranes (Bio-Rad), according to manufacturer’s instructions. The blots were then blocked using 10% low-fat milk in PBS +0.05% Tween 20 (PBS-T), reacted with the corresponding primary mAb or polyclonal serum, and then with either protein A or goat anti-mouse coupled to horseradish peroxidase (Amersham) for polyclonal rabbit serum or murine mAb, respectively. Blots were developed using the ECL detection kit (Amersham).

RNA extraction and Northern blots

Cytoplasmic RNA was extracted using the NP40-lysis protocol (Ginsberg et al., 1991). Where appropriate, cycloheximide was added to cultures to a final concentration of 15 µg/ml. Northern blots were performed as described (Ginsberg et al., 1990), using Hybond-N membranes (Amersham) for transfer. The blots were hybridized to random-primed probes in a solution containing 50% formamide at 42°C. The highest stringency of wash was 0.2×SSPE, 0.1% SDS, at 60°C.

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