MDM2 Is a Target of Simian Virus 40 in Cellular Transformation and during Lytic Infection

WILHELM HENNING, GABOR ROHALY, THOMAS KOLZAU, UWE KNIPPSCHILD,[†]
HEIKO MAACKE, AND WOLFGANG DEPPERT*

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, D-20251 Hamburg, Germany

Received 19 March 1997/Accepted 8 July 1997

Phosphopeptide analyses of the simian virus 40 (SV40) large tumor antigen (LT) in SV40-transformed rat cells, as well as in SV40 lytically infected monkey cells, showed that gel-purified LT that was not complexed to p53 (free LT) and p53-complexed LT differed substantially in their phosphorylation patterns. Most significantly, p53-complexed LT contained phosphopeptides not found in free LT. We show that these additional phosphopeptides were derived from MDM2, a cellular antagonist of p53, which coprecipitated with the p53-LT complexes, probably in a trimeric LT-p53-MDM2 complex. MDM2 also quantitatively bound the free p53 in SV40-transformed cells. Free LT, in contrast, was not found in complex with MDM2, indicating a specific targeting of the MDM2 protein by SV40. This specificity is underscored by significantly different phosphorylation patterns of the MDM2 proteins in normal and SV40-transformed cells. Furthermore, the MDM2 protein, like p53, becomes metabolically stabilized in SV40-transformed cells. This suggests the possibility that the specific targeting of MDM2 by SV40 is aimed at preventing MDM2-directed proteasomal degradation of p53 in SV40-infected and -transformed cells, thereby leading to metabolic stabilization of p53 in these cells.

The transforming activity of the major transforming protein of simian virus 40 (SV40), the large tumor antigen (LT), results mainly from its specific interactions with a variety of cellular proteins (reviewed in reference 46). Among these, the interactions of LT with the tumor suppressor proteins pRb (and pRb-related proteins) and p53 are the best characterized (reviewed in references 33 and 44). A major function of these interactions is to abolish the tumor suppressor functions of these proteins (reviewed in references 44, 65, and 76). However, at least the interaction of LT with p53 seems to be more complex, because there is evidence for an auxiliary transforming function of p53 in SV40 transformation (16, 49, 73, 74, 81). This auxiliary function of p53 is tightly connected with metabolic stabilization of p53, leading to strongly enhanced p53 levels in SV40-transformed cells. During cellular transformation, metabolic stabilization of p53 and the ensuing increase in p53 levels correlate with an enhanced transformation frequency and progression to a more highly transformed phenotype (16, 73, 74, 80, 81). Metabolic stabilization of p53 during SV40 transformation of nonpermissive rodent cells is a cellular event, which is mediated by the cooperative action of LT and the second transforming protein of SV40, the small tumor antigen (small t) (74). Stabilization of p53 complexed with LT thus might be an important aspect of the small t helper function in SV40 transformation (reviewed in reference 80). From these data, we concluded that targeting of p53 during SV40 transformation might play a dual role, not simply leading to the inactivation of the tumor suppressor functions of p53 but also endowing the metabolically stabilized p53 with an important auxiliary function in SV40 transformation. The postulated auxiliary effect of metabolically stabilized p53 could be a function of the stabilized p53 itself, acting like a “gain-of-function”

mutant p53 (reviewed in references 18 and 19), or a property of the LT-p53 complex. The latter possibility recently has been suggested by our finding that p53, by complexing with LT, seems to “activate” the transforming potential of the C-terminal transforming domain of LT (81). Metabolic stabilization of p53 in SV40 transformation thus could be regarded as a means of substantially increasing the fraction of such “activated,” i.e., fully transformation-competent, LT in complex with p53.

LT is a phosphoprotein, and the regulatory role of phosphorylation in modulating the various activities of this protein has been well established (reviewed in references 23 and 64). We therefore analyzed whether LT complexed with p53 and free LT might differ in their phosphorylation states. Such a difference might provide an indication for explaining the possibly different activities of these LT subpopulations. Our analyses of the phosphorylation status of p53-complexed LT and of free LT in SV40-transformed rat cells led to the discovery that MDM2, a physiological antagonist of p53 (2, 52, 57, 58, 60), is a target of SV40 in cellular transformation and also during lytic infection: MDM2 was coprecipitated with LT-p53 complexes and quantitatively complexed with the free p53 in SV40-transformed cells. Furthermore, the MDM2 protein in SV40-transformed cells exhibited a significantly different phosphorylation pattern from the MDM2 in nontransformed cells and, like p53 in SV40-transformed cells, became metabolically stabilized.

Since MDM2 was recently shown to direct p53 to proteasomal degradation (29, 39), the targeting of MDM2 by SV40 might serve to achieve metabolic stabilization of p53 in SV40-transformed and -infected cells. Furthermore, since MDM2, aside from its ability to negatively regulate p53 activities and to direct p53 into the degradation pathway, seems to have an oncogenic potential of its own (24, 60), the specific targeting of MDM2 to free p53 and LT-p53 complexes could provide a basis for the auxiliary function of metabolically stabilized p53 in SV40 transformation.

(This work was conducted by W. Henning in partial fulfillment of the requirements for a Ph.D. at the University of Hamburg, Hamburg, Germany.)

* Corresponding author. Mailing address: Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Martinistr. 52, D-20251 Hamburg, Germany. Phone: 40-48051-261. Fax: 40-48051-117. E-mail: deppert@hpi.uni-hamburg.de.

[†] Present address: Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee DD1 9SY, United Kingdom.

MATERIALS AND METHODS

Cells and virus infection. The cells used in this study were normal Fisher rat fibroblasts REF52 (6) and F111 (25), SV40-transformed REF52 rat fibroblasts (SV52) (6), Swiss 3T3 (75) and SV40-transformed Swiss SV3T3 mouse fibroblasts, and an African green monkey kidney cell line (CV1) (36). All cells were grown at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Monolayer cultures of CV1 monkey cells were inoculated with wild-type SV40 (strain 776) at an input multiplicity of infection of 10. After an adsorption period of 1 h at 37°C, unabsorbed virus was removed and growth medium was added to the culture. The cells were analyzed 48 h postinfection.

Labeling of cells. Subconfluent cells were labeled with [³⁵S]cysteine-[³⁵S]methionine (60 μ Ci ml⁻¹) (Translabel; ICN) for 60 min at 37°C in methionine- and cysteine-free DMEM supplemented with 10% dialyzed FCS. Alternatively, the cells were radiolabeled for 3 h at 37°C with ³²P_i (0.5 mCi ml⁻¹) (carrier free; Hartmann Analytics) in P_i-free DMEM (ICN) supplemented with 10% P_i-free FCS. In each case, the labeling medium was pre-equilibrated overnight at the labeling temperature.

Lysis, immunoprecipitation, SDS-PAGE, and Western immunoblotting. ³⁵S-treated cells were washed with ice-cold PBS and extracted for 30 min at 4°C with lysis buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM dithiothreitol, 1 mM EGTA, 30 μ g of aprotinin (200 kIU) (Trasylol; Bayer) per ml, 50 μ g of leupeptin per ml, and 20% immunoglobulin G-free and heat-inactivated FCS. Whole-cell extracts from ³²P-treated cells were prepared by lysis in sodium phosphate-based lysis buffer (pH 9.0). Extracts were cleared by centrifugation for 45 min at 12,000 $\times$ g at 4°C. Proteins were immunoprecipitated with the monoclonal antibody PAb419, specific for LT (28) or PAb421, specific for p53 (28), or with an MDM2 antiserum (38) and 50 μ l of protein A-Sepharose for 2 h at 4°C on a rotating shaker. The immunoprecipitates were washed five times with phosphate-buffered saline (PBS) containing 1% Nonidet P-40, three times with PBS, and once with 0.1 $\times$ PBS. Then immune complexes were denatured in sodium dodecyl sulfate (SDS) sample buffer, and radiolabeled proteins were separated on SDS-polyacrylamide gels and detected by fluorography and autoradiography, as described previously (54).

The amounts of immunoprecipitated MDM2 were determined by Western blotting. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and visualized by enhanced chemiluminescence (Amersham). Protein bands corresponding to MDM2 were detected by use of an MDM2 antiserum (primary antibody) and horseradish peroxidase-conjugated mouse anti-guinea pig immunoglobulin G (secondary antibody).

Pulse-chase analysis. Equal numbers of subconfluent cells were pulse-labeled for 15 min with [³⁵S]cysteine-[³⁵S]methionine (60 μ Ci ml⁻¹) in methionine- and cysteine-free DMEM containing 10% dialyzed FCS and chased with growth medium for 15 to 240 min. Whole-cell extracts were prepared by lysis in Tris-based lysis buffer (see above).

Two-dimensional phosphopeptide analysis. Immunoprecipitated proteins were purified by SDS-PAGE on 1-mm-thick 7.5% acrylamide gels. They were then extracted from the gels and oxidized with performic acid as described previously (37).

Phosphopeptides of SV40 LT and MDM2 were prepared by sequential digestion with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) and pronase E (Sigma) and were separated and analyzed on thin-layer plates by electrophoresis at pH 1.9 for 45 min and 1 kV followed by ascending chromatography in isobutyric buffer by the method of Scheidtmann et al. (67) and Knippschild et al. (37). The phosphopeptides were visualized by autoradiography.

In vitro transcription, in vitro translation, and in vitro binding assays. The in vitro transcription and in vitro translation assays were carried out as previously described (55). Briefly, a full-length mouse wt p53 cDNA clone (8), a mouse MDM2 cDNA clone (38), and an SV40 LT cDNA clone (10) were transcribed in vitro with T7 and T3 RNA polymerases by using the Riboprobe Gemini transcription system (Promega). In vitro translation of p53, SV40 LT, and MDM2 was performed in the presence of 30 μ Ci of ³⁵S-labeled or unlabeled methionine and cysteine with a rabbit reticulocyte lysate system (Promega). A 5- μ l volume of each reticulocyte lysate containing newly synthesized proteins was diluted in lysis buffer (see above), labeled and unlabeled proteins were mixed, and the mixture was incubated for 1 h at 4°C. Unlabeled MDM2 and LT then were immunoprecipitated with the respective antibodies. Immunocomplexes were separated by SDS-PAGE, and associated labeled proteins were detected by fluorography.

RESULTS

Free LT and p53-complexed LT differ in their phosphorylation patterns. To analyze whether p53-complexed LT and free LT might differ in their phosphorylation states, we isolated p53-complexed LT and free LT by sequential immunoprecipitations. SV40-transformed REF52 rat fibroblast cells (SV52) were metabolically labeled either with [³⁵S]methionine-

FIG. 1. Analysis of SV40-transformed cells for LT-p53 complexes and for free LT. Cells were radiolabeled either with [³⁵S]cysteine-[³⁵S]methionine (A) or ³²P_i (B). Aliquots of whole-cell extracts were immunoprecipitated for total LT (lanes 1), LT-p53 complex (lanes 2 to 4), and free LT (lanes 5) with the respective monoclonal antibodies. Free LT was purified by clearing the extract of p53-LT complexes by three sequential immunoprecipitations with PAb421 (lanes 2 to 4) followed by immunoprecipitation with PAb419, specific for LT. The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel and subjected to fluorography or autoradiography, respectively.

online-[³⁵S]cysteine for 1 h or with ³²P_i for 3 h, as described in Materials and Methods. Aliquots of whole-cell extracts from these cells were immunoprecipitated with monoclonal antibody PAb419, recognizing an N-terminal epitope of LT (53), to obtain total (i.e., free and complexed) LT, or with PAb421, recognizing a C-terminal epitope of p53 (42), to specifically precipitate LT complexed to p53. To purify uncomplexed (free) LT, these extracts were cleared of p53-LT complexes by three sequential immunoprecipitations with the p53-specific PAb421, and free LT was immunoprecipitated from the cleared extracts with PAb419. Analysis of the ³⁵S-labeled proteins by SDS-PAGE (Fig. 1A) demonstrated that in these cells about half of the total LT was complexed with p53 (compare lanes 1, 2, and 5). In agreement with previous reports (22, 72), analysis after ³²P labeling showed that LT complexed with p53 was phosphorylated to a significantly higher degree than was free LT (Fig. 1B, compare lanes 2 and 5). Total LT, p53-complexed LT, and free LT were gel purified and then subjected to two-dimensional phosphopeptide mapping analysis, as described previously (37) and in Materials and Methods. Figure 2A shows the phosphopeptide pattern of free LT, displaying the phosphorylation sites of LT as determined in several laboratories (17, 32, 37, 67, 69). Although the phosphopeptide pattern of p53-complexed LT (Fig. 2C) was similar to that of free LT (Fig. 2A), some striking differences were observed. The most prominent was the appearance of phosphopeptides not present in free LT (I to VIII in Fig. 2C). In addition, several phosphopeptides of free LT were reduced or not present in p53-complexed LT (e.g., peptides 4, 6a, 6, 11, 12a, and d). Whereas the loss of phosphopeptides could be easily explained by an altered phosphorylation of p53-complexed LT, the appearance of additional phosphopeptides in p53-complexed LT posed a problem insofar as we considered it very unlikely that such a major phosphorylation site as represented, e.g., by peptide I in Fig. 2C would have been missed in previous analyses. Phosphopeptide analysis of total LT (Fig. 2B) shows that the additional phosphopeptides present in the map of p53-complexed LT could also be detected in the phosphopeptide map of total LT when it was overexposed (compare the relative intensities of the LT-specific peptide 13 in

FIG. 2. Phosphopeptide analysis of free LT, large T complexed to p53, total LT, and MDM2 in SV52 cells. The cells were labeled with $^{32}\text{P}_i$ and free LT (A), total LT (B), large T complexed to p53 (C), and MDM2 (D) were immunoprecipitated from whole-cell extracts as described in the legend to Fig. 1 and 3. Phosphopeptides of LT and MDM2 were prepared by sequential digestion of purified proteins with TPCK-treated trypsin and pronase E, followed by two-dimensional analysis by electrophoresis (first dimension) and ascending chromatography (second dimension) as described in Materials and Methods. The LT phosphopeptides were numbered by the system of Knippschild et al. (37). The origin is marked with a solid square, the anode is on the left, and P_i indicates the position of free inorganic phosphate. Additional peptides in total LT and the LT complexed to p53 and phosphopeptides derived from immunopurified total MDM2 are indicated as I to IX.

Fig. 2A, B, and C). This finding made it difficult to assume that these additional peptides resulted from artifacts generated either during immunoprecipitation or during peptide mapping.

MDM2 coprecipitates with LT-p53 complexes and with free p53. As a possible explanation for the presence of additional phosphopeptides in the peptide map of p53-complexed LT, we considered the specific coprecipitation of a protein with an apparent molecular mass on SDS-PAGE of 90 kDa with the p53-LT complex. The MDM2 protein appeared to be a promising candidate for such a protein. MDM2 is a physiological p53 binding protein (2, 29, 39, 52, 57, 58, 60), which might interact with p53 in the LT-p53 complex or with the free p53 present in SV40-transformed cells, and it would comigrate with LT, since it, like LT, has an apparent molecular mass of 90 kDa on SDS-PAGE. MDM2 associated with the free p53 in SV52 cells also would coprecipitate with the LT-p53 complex, since isolation of this complex from SV52 cells by immunoprecipitation of the cellular extract with PAb421 will precipitate p53 both in complex with LT and free. To test this possibility, extracts of SV52 cells, labeled either with [^{35}S]methionine- ^{35}S]cysteine or with $^{32}\text{P}_i$, were cleared of LT and LT-p53 complexes by sequential immunoprecipitation with PAb419. The cleared extracts were then immunoprecipitated with PAb421, which then recovered only any "free" p53 still in the extract (14, 15). The result of this analysis is shown in Fig. 3.

FIG. 3. Analysis of SV52 cells for free p53, MDM2 complexed to p53, and MDM2. The cells were labeled with either [^{35}S]cysteine- ^{35}S]methionine (A) or $^{32}\text{P}_i$ (B) and extracted, and free p53 (lanes 4) was immunopurified by clearing the extracts of LT and LT-p53 complexes by three sequential immunoprecipitations with PAb419 (lanes 1 to 3) followed by precipitation with PAb421 (lanes 4). MDM2 was immunopurified by with a polyclonal MDM2 antiserum (lanes 5). Immunoprecipitates were analyzed on SDS-11.5% polyacrylamide gels and subjected to fluorography and autoradiography, respectively.

FIG. 4. Phosphopeptide analysis of MDM2 coprecipitating with free p53 and of MDM2 precipitated with an anti-MDM2 serum from SV52 cells. SV52 cells were labeled with ^{32}P . Total MDM2 (A) and MDM2 complexed to free p53 (B) were immunopurified from whole-cell extracts as described in the legend to Fig. 3 and subjected to two-dimensional phosphopeptide mapping analysis. The origin is indicated by a solid square, and the anode is on the left. Phosphopeptides derived from MDM2 are indicated as I to IX, as Fig. 2D.

Lanes 1 to 3 show the clearing of the extracts from LT and LT-p53 complexes by sequential immunoprecipitation with PAb419, and lane 4 shows the free p53 recovered from the cleared extracts by immunoprecipitation with PAb421. Only a small fraction (approximately 1%) of the total p53 present in the extracts of SV52 cells was found not to be complexed with LT. This p53 appeared to be phosphorylated to a significantly lesser extent than was the p53 present in LT-p53 complexes, since it was hardly detected after ^{32}P labeling (Fig. 3B). A faint band at an apparent molecular mass of 90 kDa, which could represent MDM2 coprecipitating with the free p53, was seen after both ^{35}S and ^{32}P labeling. To test this assumption, extracts of SV52 cells were immunoprecipitated with a polyclonal antibody raised against purified, bacterially expressed mouse MDM2 protein (38). Figure 3, lanes 5, shows the presence of a 90-kDa protein comigrating with LT, as well as a 90-kDa protein coprecipitating with free p53 and an 85-kDa protein, most probably representing the MDM2 90- and 85-kDa doublet (4, 59). Phosphopeptide mapping of the 90-kDa protein precipitated by the MDM2 antiserum from the whole-cell extract of ^{32}P -labeled cells (Fig. 4A) and of the 90-kDa protein coprecipitating with free p53 (Fig. 4B) showed a close similarity, indicating that these proteins were related. Therefore, we concluded that these 90-kDa proteins represented MDM2.

The phosphopeptide map of MDM2 precipitated from the whole-cell extract of SV52 cells by the MDM2 antiserum is also shown in Fig. 2D and demonstrates that the additional phosphopeptides I to VIII, present in the phosphopeptide map of p53-complexed LT, were derived from MDM2 coprecipitating with the LT-p53 complex. Comparison of Fig. 4A and B showed some differences in the phosphorylation patterns of MDM2 in complex with free p53 (Fig. 4B) and of MDM2 precipitated by the MDM2 antiserum (Fig. 4A). The most prominent difference was the absence of peptide IX in MDM2 complexed to p53. A similar difference can also be seen in the phosphopeptide maps of MDM2 precipitated by the MDM2 antiserum (Fig. 2D) and MDM2 coprecipitated with the LT-p53 complex (Fig. 2C) in SV52 cells, suggesting that the respective MDM2 proteins are differently phosphorylated.

MDM2 is targeted by SV40 during lytic infection. The results shown above suggested that MDM2 might be a target of SV40 in cellular transformation. To analyze whether the targeting of MDM2 by LT-p53 complexes is a general property of SV40-host interactions, we asked whether MDM2 might also interact with LT-p53 complexes during SV40 lytic infection. CV1 monkey cells were infected with SV40 at a multiplicity of infection of 10. At 45 h postinfection, the cells were labeled with ^{32}P for 3 h and extracted. Free LT and p53-complexed LT were immunopurified as described above, subjected to phosphopeptide analysis, and analyzed for the possible association of MDM2. The free LT in these cells exhibited the phosphopeptide pattern characteristic of LT (Fig. 5A; compare Fig. 2A). The phosphopeptide pattern of p53-complexed LT in SV40-infected CV1 cells (Fig. 5B) resembled that of p53-complexed LT in SV52 cells (Fig. 2C). Again, several LT-specific phosphopeptides were reduced compared to those for free LT (e.g., peptides 4, 6a, 12a, c, and e), and the additional phosphopeptides (I to VIII) indicated the coprecipitation of MDM2 with the p53-LT complex. Figure 5C shows the phosphopeptide map of MDM2 precipitated with the MDM2 antiserum. Comparison with Fig. 5B shows that the MDM2 protein in the LT-p53 complex from SV40-infected CV1 cells, like the one in SV52 cells, was somewhat differently phosphorylated from the MDM2 protein precipitated with the MDM2 antiserum. The most significant difference was the presence of an additional peptide (IX) in MDM2 precipitated from whole-cell extracts by the MDM2 antiserum. Furthermore, peptides IV and VII are more abundant relative to peptides V and VIII in MDM2 precipitated with the MDM2 antiserum than in MDM2 associated with the LT-p53 complex.

MDM2 forms a trimeric complex with LT and p53 in vivo and in vitro. The phosphopeptide maps of free LT in SV40-transformed and -infected cells (Fig. 2A and 5A) did not contain any MDM2-specific phosphopeptides. Conversely, the analysis of the phosphopeptide maps of the MDM2 proteins precipitated with the MDM2 antiserum from whole-cell extracts of either SV40-transformed (Fig. 2D and 4A) or SV40 lytically infected cells (Fig. 5C) did not show any of the LT-specific phosphopeptides, indicating that LT did not coprecipitate with MDM2 in these immunoprecipitations. These findings could mean that MDM2 in SV40-infected and -transformed cells is associated exclusively with the free p53 coprecipitating with the LT-p53 complex. This interpretation would also be supported by the fact that approximately the same amount of p53 was coprecipitated with MDM2 when the MDM2 antiserum was used (Fig. 3, lanes 5), as the amount of free p53 in SV52 cells (lanes 4). On the other hand, since immunoprecipitation of total LT with the LT-specific PAb419 will not coprecipitate any free p53, this interpretation is not compatible with our finding that the phosphopeptide map of

FIG. 5. Phosphopeptide analysis of free LT, LT complexed to p53, and of MDM2 in CV1 cells after lytic infection with SV40. The cells were labeled with $^{32}\text{P}_i$, and whole-cell extracts were prepared by lysis in sodium phosphate-based lysis buffer (pH 9.0). (A) Free LT was obtained by clearing the extract of p53-LT complexes by three sequential immunoprecipitations with PAb421 followed by immunoprecipitation with PAb419; (B) LT complexed to p53 was coprecipitated with PAb421; (C) MDM2 was immunoprecipitated with the polyclonal MDM2 antiserum. Phosphopeptides were prepared as described in the legend to Fig. 2. The origin is marked by a solid square, the anode is on the left, and free radioactive phosphate is indicated by P_i . The LT-specific phosphopeptides were numbered by the nomenclature of Knippschild et al. (37). Phosphopeptides derived from MDM2 are indicated as I to IX as in Fig. 2D and 4A.

total LT in SV52 cells also contained MDM2-specific peptides (Fig. 2B). As an alternative explanation, we therefore considered the possibility that the MDM2 associated with the LT-p53 complex was not recognized by our antiserum. To test this

FIG. 6. In vitro association of MDM2 with LT and with LT-p53 complexes. MDM2, LT, and p53 were transcribed in vitro from cDNA clones and translated in vitro by the Promega system as described in Materials and Methods. A 5- μl volume of the 50- μl in vitro translation mixture was diluted in Tris-based lysis buffer, ^{35}S -labeled and unlabeled proteins were mixed, the mixtures were incubated for 1 h, and the unlabeled proteins were immunoprecipitated with the respective antibodies. Lanes: 1, control, analysis of nonspecific binding of ^{35}S -labeled p53, LT, and MDM2 to protein A-Sepharose; 2, precipitation of unlabeled MDM2 and ^{35}S -labeled LT with polyclonal MDM2 antiserum; 3, precipitation of unlabeled MDM2 and ^{35}S -labeled LT and p53 with polyclonal MDM2 antiserum; 4, precipitation of unlabeled LT and ^{35}S -labeled MDM2 and p53 with PAb 419 (p53 ΔC is a prematurely terminated, C-terminally truncated in vitro translation product [55]); 5, association of unlabeled MDM2 immunoprecipitated from whole-cell extracts of Swiss 3T3 cells, with in vitro translated, ^{35}S -labeled LT. Immunoprecipitates were analyzed on SDS-11.5% polyacrylamide gels and subjected to fluorography.

possibility, whole-cell extracts of ^{32}P -labeled SV52 cells were first cleared of MDM2 immunoprecipitable with MDM2 antiserum by three sequential immunoprecipitations. This cleared extract was subsequently immunoprecipitated for LT-p53 complexes with PAb421. p53-complexed LT then was gel purified and analyzed for the presence of coprecipitated MDM2 by phosphopeptide mapping. A phosphopeptide map virtually identical to that shown in Fig. 2C was obtained (data not shown), providing strong evidence that MDM2 is associated with the LT-p53 complex but that this MDM2 is not recognized by our MDM2 antiserum.

To analyze the possible interactions of MDM2 with free LT and with LT complexed to p53, we performed in vitro association studies with SV40 LT, mouse p53, and mouse MDM2, translated in vitro from their respective full-length mRNAs in rabbit reticulolysates (see Materials and Methods). In vitro translation allowed the selective labeling of each of the possible interaction partners with [^{35}S]methionine-[^{35}S]cysteine and, after mixing the translation reaction mixtures, immunoprecipitation of the formed complexes with either an LT- or an MDM2-specific antibody. By precipitation with the antibody against the unlabeled protein, associated ^{35}S -labeled proteins could be detected by coprecipitation. The result of this analysis is shown in Fig. 6. After mixing unlabeled in vitro-translated MDM2 with ^{35}S -labeled LT, the MDM2 antiserum was able to coprecipitate LT (Fig. 6, lane 2), indicating that in vitro MDM2 can associate with LT alone, as previously described (9). Mixing unlabeled MDM2 with labeled LT plus labeled p53 and immunoprecipitating with the MDM2 antiserum resulted in coprecipitation of both LT and p53 (lane 3), indicating the formation of a trimeric complex in vitro. Similarly, mixing unlabeled LT with labeled MDM2 and labeled p53 followed by immunoprecipitation with the LT-specific PAb419 led to coprecipitation of MDM2 and p53 (lane 4). This analysis thus yielded two important results. First, MDM2 and LT by themselves can form a complex in vitro (lane 2). This conclusion was further corroborated by mixing in vitro-translated, ^{35}S -labeled LT with an unlabeled whole-cell extract from Swiss 3T3 cells, which express relatively large amounts of MDM2 (54, 59) (see below). Precipitation of this unlabeled MDM2 with the MDM2 antiserum coprecipitated the added labeled LT (lane 5). In contrast to this in vitro association of MDM2 with LT, our

analyses gave no indication that MDM2 and free LT will form a complex *in vivo*. Second, MDM2 is also able to form a trimeric complex with LT and p53 *in vitro*, as already suggested by our *in vivo* analyses. However, in contrast to the trimeric complex formed *in vivo*, the trimeric complex formed *in vitro* was recognized by our anti-MDM2 serum.

SV40 alters the phosphorylation status of MDM2. The *in vitro* analysis of the interactions of MDM2 with LT had shown that the trimeric complex formed by LT, p53, and MDM2 *in vitro* was recognized by our MDM2 antiserum whereas such a trimeric complex formed *in vivo* was not. Furthermore, MDM2 formed a complex with free LT *in vitro* whereas no such complex could be detected *in vivo*. These differences could be simply due to structural differences in the respective complexes, e.g., differences in the stoichiometry of the reacting partners. As an alternative explanation, we considered the possibility that these differences reflected a specific targeting of MDM2 by SV40 in cellular transformation and in lytic infection, aimed at preventing the association of MDM2 with free LT and furthering its specific association with free p53 and LT-p53 complexes *in vivo*. Such an SV40-induced altered specificity of the interactions of MDM2 could be achieved by an altered conformation of MDM2 in SV40-infected and -transformed cells, which might also account for the loss of recognition of MDM2 associated with LT-p53 complexes by our MDM2 antiserum.

To get an indication of whether SV40 transformation might alter the conformational status of MDM2, we wanted to analyze the phosphorylation pattern of MDM2 from normal (F111 and REF52) and SV40-transformed (SV52) rat fibroblasts. Such an analysis, however, was not possible, since F111 and REF52 cells contained too little ^{32}P -labeled MDM2 protein, probably reflecting the fact that these cells contain very little MDM2 protein (see below). We therefore analyzed the MDM2 proteins from Swiss 3T3 cells and from an SV40-transformed derivative of these cells (Swiss SV3T3 cells), since MDM2 is abundantly expressed in these cells (54, 59) (see below). Swiss 3T3 and Swiss SV3T3 cells were labeled with $^{32}\text{P}_i$ for 3 h and extracted. The extracts were precipitated with MDM2 antiserum, and the gel-purified MDM2 proteins were analyzed by peptide mapping. Figure 7 shows the complex phosphopeptide patterns of the MDM2 proteins from SV40-transformed (Fig. 7A) and normal (Fig. 7B) Swiss 3T3 cells. Despite some similarities, the respective phosphorylation patterns were grossly different. A mixing experiment (Fig. 7C) involving MDM2 phosphopeptides from Swiss 3T3 and from Swiss SV3T3 cells revealed that MDM2 from SV40-transformed Swiss SV3T3 cells contained at least four phosphopeptides not found in normal Swiss 3T3 cells (peptides II, III, V, and VIII). On the other hand, MDM2 from Swiss 3T3 cells contained at least one phosphopeptide (peptide X) not found in MDM2 from Swiss SV3T3 cells. In addition to these qualitative alterations, several peptides common to MDM2 in Swiss 3T3 cells and MDM2 in Swiss SV3T3 cells (peptides XI, XII, XIII, XIV, XV, and XVI) were phosphorylated to a significantly higher degree in MDM2 from normal Swiss 3T3 cells than in MDM2 from Swiss SV3T3 cells. Our conclusion that SV40 is able to induce a profound alteration in MDM2 phosphorylation is further supported by our finding that virtually identical results were obtained in a phosphopeptide analysis of MDM2 from normal BALB/c 3T3 cells and from BALB/c SV3T3 cells (data not shown) and the finding that peptides II, III, V, and VIII (absent in MDM2 from normal cells) were present in MDM2 from SV40 lytically infected cells and in MDM2 from all SV40-transformed cells analyzed so far.

FIG. 7. Comparison of the phosphopeptide patterns of MDM2 in SV40-transformed and normal cells. MDM2 was immunopurified from Swiss SV3T3 (A) and Swiss 3T3 (B) cells labeled with $^{32}\text{P}_i$, and MDM2-specific phosphopeptides were prepared and analyzed. The peptides prepared for analysis in panels A and B were mixed and then separated in two dimensions to indicate comigrating peptides (C). The origin is marked with a solid square, the anode is on the left, and P_i indicates the position of free inorganic phosphate. MDM2-specific peptides are indicated as I to XXI.

MDM2 becomes metabolically stabilized during SV40 transformation. Recently, it has been shown that MDM2 controls the level of p53 in normal cells by channeling p53 into the proteasomal degradation pathway (29, 39). In SV40-infected

FIG. 8. Steady-state levels of MDM2 in normal and in SV40-transformed fibroblasts. Whole-cell extracts from F111 (lane 1), REF52 (lane 2), SV52 (lane 3), and Swiss 3T3 (lane 4) cells were immunoprecipitated for MDM2 with a polyclonal anti-MDM2 antibody. The immunoprecipitates were separated on an SDS-11.5% polyacrylamide gel, and proteins were transferred to nitrocellulose by electroblotting. The polyclonal MDM2 antibody was used for subsequent chemiluminescence detection.

and -transformed cells, p53 becomes metabolically stabilized by a virally induced cellular event (16, 73, 74, 81). The specific targeting of a posttranslationally modified MDM2 to p53 and LT-p53 complexes in SV40-infected and -transformed cells therefore raised the possibility that metabolic stabilization of p53 in SV40 infection and transformation and the targeting of MDM2 were connected. Since MDM2 is short-lived in normal cells, with a half-life similar to that of p53 (59), we first asked whether the MDM2 proteins in normal and in SV40-transformed cells might have different turnover rates.

With a few exceptions (e.g., Swiss 3T3 cells [see below]), MDM2 is a protein of very low abundance in normal cells. This is shown in Fig. 8, representing an analysis of the MDM2 content in normal rat F111 (lane 1) and REF52 (lane 2) fibroblasts, as well as in SV52 cells (lane 3), a derivative of REF52 cells (6). For comparison, the MDM2 content of Swiss 3T3 cells is shown in lane 4. A total of 5×10^7 cells were extracted, MDM2 was immunoprecipitated with our MDM2 antiserum, and the amount of MDM2 present in these cells was determined by Western blotting. Using this approach, we were unable to demonstrate the presence of MDM2 protein in F111 and REF52 cells (lanes 1 and 2, respectively). However, after metabolic labeling of these cells with $^{32}\text{P}_i$, a small amount of MDM2 protein could be visualized, demonstrating that these cells express MDM2, albeit at a very low level (data not shown). In SV52 cells, Western blotting detected a faint MDM2 band (lane 3), indicating an increase in MDM2 levels in these cells over nontransformed rat fibroblasts. However, the amount of MDM2 in SV52 cells was still far smaller than that in Swiss 3T3 cells (lane 4).

This increase in MDM2 levels in SV52 cells was not due to enhanced *mdm2* gene transcription, since the *mdm2* mRNA levels in REF52 and in SV52 cells were similar (data not shown). We therefore considered metabolic stabilization of MDM2 as a means of elevating MDM2 levels in SV52 cells. MDM2 in normal cells is unstable, with a half-life of about 20 min (59). This is also shown in Fig. 9A for the MDM2 protein in Swiss 3T3 cells, which were used instead of REF52 and F111 cells, because the last two cell lines do not contain enough MDM2 for such an analysis (see above). The major MDM2 band seen after a 15-min pulse (Fig. 9A, lane p) decreased during the ensuing chase periods of 15, 30, and 60 min (lanes c_1 to c_3), with the MDM2 protein being barely visible after the 60-min chase. The MDM2 protein in Swiss SV3T3 cells, in contrast, was stable during these chase periods (Fig. 9B). Likewise, the MDM2 protein in SV52 cells was stable during a 2-h chase period following a 15-min pulse-label (Fig. 9C, lanes p and c_1). An approximate 50% decrease in the intensity of the MDM2 band was seen after a 4-h chase period (Fig. 9C, lane c_2), indicating a half-life of the MDM2 protein in SV52 cells of about 4 h, which represents a significant stabilization of this protein after SV40 transformation.

FIG. 9. Half-life of MDM2 in Swiss 3T3, Swiss SV3T3, and SV52 cells. The cells were pulse labeled (p) and pulse-chase labeled with 60 μCi of [^{35}S]cysteine- ^{35}S methionine per ml. (A) Swiss 3T3 cells, 15-min pulse, and 15-min (c_1), 30-min (c_2), and 60-min (c_3) chase times; (B) Swiss SV3T3 cells, 15-min pulse, and 15-min (c_1), 30-min (c_2), and 60-min (c_3) chase times; (C) SV52, 15-min pulse and 120-min (c_1) and 240-min (c_2) chase times. Whole-cell extracts were immunoprecipitated for MDM2, and the immunoprecipitates were analyzed on SDS-11.5% polyacrylamide gels and subjected to fluorography.

DISCUSSION

The *mdm2* oncogene was originally discovered due to its gene amplification in a spontaneously transformed BALB/c 3T3 cell line (21). Overexpression of *mdm2* led to immortalization of rat embryo fibroblasts and, in conjunction with the expression of an activated *ras* oncogene, to phenotypic transformation of these cells (24). The oncogenic potential of the *mdm2* gene is underscored by the finding that the human *mdm2* gene is overexpressed in a significant number of human tumors, including soft tissue sarcomas, osteosarcomas, gliomas, and leukemias (13, 40, 43, 57, 66). Overexpression of *mdm2* in these tumors is due mostly to gene amplification but can also occur by other means, such as translocation of the *mdm2* gene (7), enhanced mRNA levels (11), and enhanced translation (41).

The molecular basis of the oncogenic activity of the MDM2 protein is not yet understood in detail. Best known is its function as a negative regulator of the p53 tumor suppressor protein. MDM2 can form a tight complex with p53 and block the ability of p53 to transcriptionally activate gene expression (12, 52, 58). Since the *mdm2* gene itself is an important target of transcriptional activation by p53 (3, 27, 35, 60, 79), expression of both genes is controlled by an autoregulatory feedback loop (61, 77), and negative regulation of p53 has been shown to be essential for embryonic survival in mice (34, 45). However, the MDM2 protein has additional functions which might contribute to its oncogenic potential. MDM2 binds the retinoblastoma gene product pRb, thereby possibly relieving the transcriptional activator E2F1 from negative regulation by pRb (78). In addition, MDM2 binds directly to the E2F1/DP1 transcription factor and stimulates its transcriptional activity (48). MDM2 also forms a tight complex in vivo with the L5 ribosomal pro-

tein, which in turn is associated with 5S rRNA (47). Furthermore, the MDM2 protein binds directly to RNA, since it efficiently interacts with poly(G) homopolymer stretches of RNA and with specific RNA molecules in a sequence- and structure-specific manner via its RING finger domain (20). So far, however, neither the function of the MDM2/L5 complex nor that of the RNA binding properties of MDM2 is understood.

In this study, we provide evidence that the MDM2 protein is targeted by SV40 during lytic infection and in cellular transformation, since the MDM2 protein specifically coprecipitates with LT-p53 complexes and binds all the free p53 in these cells. The interaction of MDM2 with LT-p53 complexes probably had not been recognized before because of the identical apparent molecular masses of LT and MDM2 of about 90 kDa in SDS-PAGE and because the MDM2 protein is of low abundance even in SV40-infected or -transformed cells. However, it should be acknowledged that Tack and Wright (72) previously had suggested the coprecipitation of a cellular phosphoprotein of 90 kDa with the LT-p53 complex in SV40 lytically infected monkey cells. The targeting of MDM2 by SV40 is specific, since MDM2 specifically associates with LT-p53 complexes and with free p53 but not with free LT. Furthermore, MDM2 in SV40-transformed cells becomes metabolically stabilized and displays a significantly altered phosphorylation pattern from that in normal or uninfected cells. Although we showed the specific targeting of MDM2 by SV40 only for a very limited selection of cells in this study, very similar observations about altered phosphorylation and metabolic stabilization of MDM2 in a variety of SV40-transformed cells, as well as in clone 6 cells (50), supertransformed with SV40 LT (30) were made. We thus conclude that the targeting of MDM2 by SV40 is a general property of SV40 LT.

The coprecipitation of MDM2 with LT-p53 complexes was detected by analyzing the phosphopeptide map of LT complexed to p53, which revealed the presence of phosphopeptides not attributable to LT. These phosphopeptides were quite prominent, suggesting that MDM2 is a major component of the LT-p53-MDM2 complex. However, quantitative analysis of MDM2 associated with p53-complexed LT proved to be difficult, since our MDM2 antiserum failed to recognize this fraction of MDM2. Similar observations have been made with a polyclonal antiserum recognizing p300, which could not coprecipitate LT from extracts of SV40-infected cells (1). Nevertheless, one can assume that the total amount of MDM2 in SV40-infected cells and in most SV40-transformed cells is rather small and that the prominence of the MDM2 phosphopeptide spots in the phosphopeptide map of LT complexed to p53, especially that from SV40-infected cells, reflects the fact that the MDM2 protein is highly phosphorylated. Phosphorylation of MDM2 in SV40-infected and -transformed cells was quite different from that of MDM2 in normal cells, since the phosphopeptide map of MDM2 from these cells exhibited a number of additional spots, indicating phosphorylation of MDM2 at additional phosphorylation sites. We therefore assume that SV40 early gene expression leads to an altered phosphorylation state of MDM2. The altered phosphorylation of MDM2 upon SV40 infection and transformation further underscores that MDM2 is a specific target of SV40. This altered phosphorylation of MDM2 could result from an increased or a newly induced kinase activity or from the specific inhibition of a phosphatase. The activity of phosphatase 2A is affected by SV40 small t, since small t binds to phosphatase 2A (reviewed in reference 56). However, our preliminary analyses indicate that small t is not involved in altering the phosphorylation state of MDM2, since MDM2 in cells infected or transformed with an SV40 small t deletion variant exhibited the same alterations

in its phosphorylation pattern as did MDM2 in cells infected with wild-type SV40 (31). Since it was previously reported that SV40 LT induces or enhances cellular kinase activities (68, 71), it is tempting to speculate that MDM2 is a target of such an SV40 LT-induced kinase activity.

Our analyses suggest that the MDM2 protein associates both with LT-p53 complexes and with the free p53 in SV40-infected and -transformed cells. In contrast, we found no evidence for an association of MDM2 with free LT in either SV40-infected or SV40-transformed cells, since the phosphopeptide map of free LT did not contain any of the MDM2 phosphopeptides. This was a somewhat surprising finding, since SV40 LT contains the sequence TFSFLI (amino acids 217 to 222), which bears significant homology to the p53 sequence TFSDLW (human; amino acids 18 to 23) and TFSGLW (mouse; amino acids 18 to 23) mediating the association of p53 with MDM2 (62). Therefore, LT should be able to bind MDM2, and an association of LT with MDM2 had been found in insect cells doubly infected with recombinant baculoviruses coding for these proteins (63). That LT indeed has the capacity to form a complex with MDM2 is further supported by our *in vitro* association data, which clearly showed an association of the MDM2 protein with LT alone. Although we cannot rule out with certainty the possibility that phosphopeptide mapping is not sensitive enough to detect a minor association of MDM2 with free LT, we interpret the apparent lack of complex formation of MDM2 with free LT to reflect the specific targeting of MDM2 by SV40. Such a specificity is supported by the profoundly altered phosphorylation status of MDM2 after SV40 infection and transformation, which perhaps may even be causal in preventing the association of MDM2 with free LT in SV40-infected and -transformed cells. Our finding that the MDM2 in normal Swiss 3T3 cells is able to associate with LT *in vitro* (Fig. 6) is compatible with this idea.

Whereas MDM2 associated with free p53 could be quantitatively precipitated by our MDM2 antiserum, the same antibodies failed to recognize MDM2 associated with LT-p53 complexes. Again, this finding was in contrast to our *in vitro* analyses (Fig. 6), in which the trimeric complexes of MDM2 with LT and p53 were recognized equally well by the LT-specific Pab419 and the MDM2 antiserum. A possible explanation could be that these complexes differed in composition due to different stoichiometries of the individual compounds *in vitro* and *in vivo*. Whereas *in vitro* all reaction partners were present in roughly equimolar amounts, both LT and p53 in SV40-infected and -transformed cells are more abundant than MDM2. Alternatively, the *in vitro*- and the *in vivo*-formed complexes between MDM2, LT, and p53 might be qualitatively different, leading either to the blocking of epitopes recognized by the MDM2 antiserum or to the generation of an MDM2 conformation no longer recognized by the antiserum.

The critically important question regarding the targeting of MDM2 by SV40 concerns its biological relevance. Although at present we can only speculate on this topic, we consider it an intriguing finding that in SV40-transformed cells, MDM2, like p53, becomes metabolically stabilized and the posttranslationally altered MDM2 is specifically targeted to LT-p53 complexes and to free p53 but not to free LT. Our previous data about metabolic stabilization of p53 during SV40 transformation had revealed that this is a virally induced cellular process. MDM2 recently has been linked to p53 turnover insofar as it directs p53 into the proteasomal degradation pathway, thereby controlling p53 levels (29, 39). MDM2 is degraded together with p53, which explains the similar half-lives of these two proteins. It is therefore tempting to speculate that a posttranslationally altered MDM2 in SV40-transformed cells no longer

is able to enter the proteasomal degradation pathway, which also would exclude p53 from such degradation and lead to metabolic stabilization of this protein.

The stabilization of MDM2 also leads to a significant increase in MDM2 levels in these cells, thus increasing the fraction of LT-p53 complexes in a trimeric complex with MDM2 and leading to quantitative complex formation with the free p53 in these cells. The complexing of free p53 by MDM2 could be viewed simply as a means of inactivating the transcriptional activity of this p53, which would be in line with the postulated function of MDM2 as a negative regulator of p53. This interpretation, however, does not hold for the interaction of MDM2 with LT-p53 complexes, since p53 in these complexes is already transcriptionally inactivated by LT (5, 51, 70). Therefore, given the additional functions of MDM2 as outlined above, such as its ability to act as a transcriptional coactivator of the E2F1/DP1 transcription factor (48) or its ability to relieve E2F1 from negative regulation by pRb (78), one could envision an auxiliary transforming function for the targeting of MDM2 by SV40. Although this is purely speculative for the moment, such an auxiliary role would fit well with the auxiliary role postulated for metabolically stabilized p53 in SV40 transformation: metabolic stabilization of p53 enhances the fraction of LT in complex with p53, which correlates with an enhanced transforming activity of LT (81). LT-p53 complexes, in turn, are a major target of MDM2 in SV40-transformed cells, suggesting the exciting possibility that the enhanced transforming potential of LT complexed to p53 is connected to its ability to bind MDM2, perhaps by activating oncogenic functions of MDM2. However, additional studies aimed at further elucidating the role of metabolic stabilization of p53 in SV40 infection and transformation and the targeting of MDM2 by SV40 are required to substantiate this speculation.

ACKNOWLEDGMENTS

This study was supported by grants De 212/8-2 and De 212/9-3 from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. The Heinrich-Pette-Institute is supported by the Freie und Hansestadt Hamburg and by the Bundesministerium für Gesundheit. This study was greatly aided by NATO collaborative research grant 920123, furthering fruitful discussions with Gernot Walter, University of California, San Diego, Calif.

We thank Philip Lecane for critically reviewing the manuscript.

REFERENCES

- Avantaggiati, M. L., M. Carbone, A. Graessmann, Y. Nakatani, B. Howard, and A. S. Levine. 1996. The SV40 large T antigen and adenovirus E1a oncoproteins interact with distinct isoforms of the transcriptional co-activator, p300. *EMBO J.* **15**:2236–2248.
- Barak, Y., and M. Oren. 1992. Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest. *EMBO J.* **11**:2115–2121.
- Barak, Y., T. Juven, R. Haffner, and M. Oren. 1993. mdm2 expression is induced by wild-type p53 activity. *EMBO J.* **12**:461–468.
- Barak, Y., E. Gottlieb, T. Juven-Gershon, and M. Oren. 1994. Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.* **8**:1739–1749.
- Bargonetti, J., I. Reynisdottir, P. N. Friedman, and C. Prives. 1992. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. *Genes Dev.* **6**:1886–1898.
- Bauer, M., E. Guhl, M. Graessmann, and A. Graessmann. 1987. Cellular mutation mediates T-antigen-positive revertant cells resistant to simian virus 40 transformation but not to retransformation by polyomavirus and adenovirus type 2. *J. Virol.* **61**:1821–1827.
- Berberich, S., and M. Cole. 1994. The mdm-2 oncogene is translocated and overexpressed in a murine plasmacytoma cell line expressing wild-type p53. *Oncogene* **9**:1469–1472.
- Bienz, B., R. Zakut-Houri, D. Givol, and M. Oren. 1984. Analysis of the gene coding for the murine cellular tumour antigen p53. *EMBO J.* **3**:2179–2183.
- Brown, D. R., S. Deb, R. M. Munoz, M. A. Subler, and S. P. Deb. 1993. The tumor suppressor p53 and the oncoprotein simian virus 40 T antigen bind to overlapping domains on the MDM2 protein. *Mol. Cell. Biol.* **13**:6849–6857.
- Buchman, A. R., L. Burnett, and P. Berg. 1986. The SV40 nucleotide sequence, p. 799–823. In J. Tooze (ed.), *DNA tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Bueso-Ramos, C. E., Y. Yang, E. deLeon, P. McCown, S. A. Stass, and M. Albitar. 1993. The human MDM-2 oncogene is overexpressed in leukemias. *Blood* **82**:2617–2623.
- Chen, J., V. Marechal, and A. J. Levine. 1993. Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.* **13**:4107–4114.
- Cordon-Cardo, C., E. Latres, M. Drobnjak, M. R. Oliva, D. Pollack, J. M. Woodruff, V. Marechal, J. Chen, M. F. Brennan, and A. J. Levine. 1994. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res.* **54**:794–799.
- Deppert, W., and M. Haug. 1986. Evidence for free and metabolically stable p53 protein in nuclear subfractions of simian virus 40-transformed cells. *Mol. Cell. Biol.* **6**:2233–2240.
- Deppert, W., M. Haug, and T. Steinmayer. 1987. Modulation of p53 protein expression during cellular transformation with simian virus 40. *Mol. Cell. Biol.* **7**:4453–4463.
- Deppert, W., T. Steinmayer, and W. Richter. 1989. Cooperation of SV40 large T antigen and the cellular protein p53 in maintenance of transformation. *Oncogene* **4**:1103–1110.
- Deppert, W., M. Kurth, M. Graessmann, A. Graessmann, and U. Knippschild. 1991. Altered phosphorylation at specific sites confers a mutant phenotype to SV40 wild-type large T antigen in a flat revertant of SV40 transformed cells. *Oncogene* **6**:1931–1938.
- Deppert, W. 1996. Binding of MAR-DNA elements by mutant p53: possible implications for its oncogenic functions. *J. Cell. Biochem.* **62**:172–180.
- Dittmer, D., S. Pati, G. Zambetti, S. Chu, A. K. Teresky, M. Moore, C. Finlay, and A. J. Levine. 1993. Gain of function mutations in p53. *Nat. Genet.* **4**:42–46.
- Elenbaas, B., M. Dobbstein, J. Roth, and A. Levine. 1996. The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol. Med.* **2**:439–451.
- Fakhrazadeh, S. S., S. P. Trusko, and D. L. George. 1991. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.* **10**:565–569.
- Fanning, E., C. Burger, and E. G. Gurney. 1981. Comparison of T antigen-associated host phosphoproteins from SV40-infected and -transformed cells of different species. *J. Gen. Virol.* **55**:367–378.
- Fanning, E. 1992. Simian virus 40 large T antigen: the puzzle, the pieces, and the emerging picture. *J. Virol.* **66**:1289–1293.
- Finlay, C. A. 1993. The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol. Cell. Biol.* **13**:301–306.
- Freeman, A. E., H. J. Igel, and P. J. Price. 1975. Carcinogenesis in vitro. I. In vitro transformation of rat embryo cells: correlations with the known tumorigenic activity of chemicals in rodents. *In vitro (Rockville)* **2**:107–116.
- Haines, D. S., J. E. Landers, L. J. Engle, and D. L. George. 1994. Physical and functional interaction between wild-type p53 and mdm2 proteins. *Mol. Cell. Biol.* **14**:1171–1178.
- Halevy, O., D. Michalovitz, and M. Oren. 1990. Different tumor-derived p53 mutants exhibit distinct biological activities. *Science* **250**:113–116.
- Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* **39**:861–869.
- Haupt, Y., R. Maya, A. Kazaz, and M. Oren. 1997. Mdm2 promotes the rapid degradation of p53. *Nature* **387**:296–299.
- Henning, W., P. Lecane, and W. Deppert. Unpublished data.
- Henning, W., G. Rohaly, and W. Deppert. Unpublished data.
- Hoess, A., I. Moarefi, K. H. Scheidtmann, L. J. Cisek, J. L. Corden, I. Dornreiter, A. K. Arthur, and E. Fanning. 1990. Altered phosphorylation pattern of simian virus 40 T antigen expressed in insect cells by using a baculovirus vector. *J. Virol.* **64**:4799–4807.
- Hoppe-Seyler, F., and K. Butz. 1995. Molecular mechanisms of virus-induced carcinogenesis: the interaction of viral factors with cellular tumor suppressor proteins. *J. Mol. Med.* **73**:529–538.
- Jones, S. N., A. E. Roe, L. A. Donehower, and A. Bradley. 1995. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* **378**:206–208.
- Juven, T., Y. Barak, A. Zauberman, D. L. George, and M. Oren. 1993. Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. *Oncogene* **8**:3411–3416.
- Kit, S., D. S. Dubbs, R. A. DeTorres, and J. L. Melnick. 1965. Enhanced thymidine kinase activity following infection of green monkey kidney cells by simian adenoviruses, simian papovavirus SV40, and an adenovirus-SV40 "hybrid." *Virology* **27**:453–457.
- Knippschild, U., J. Kiefer, T. Patschinsky, and W. Deppert. 1991. Phenotype-specific phosphorylation of simian virus 40 *tsA* mutant large T antigens in *tsA* N-type and A-type transformants. *J. Virol.* **65**:4414–4423.
- Kolzu, T. 1996. Ph.D. thesis. University of Hamburg, Hamburg, Germany.
- Kubbutat, M. H., S. N. Jones, and K. H. Vousden. 1997. Regulation of p53 stability by Mdm2. *Nature* **387**:299–303.
- Ladanyi, M., R. Lewis, S. C. Jhanwar, W. Gerald, A. G. Huvos, and J. H.

- Healey. 1995. MDM2 and CDK4 gene amplification in Ewing's sarcoma. *J. Pathol.* **175**:211–217.
41. Landers, J. E., D. S. Haines, J. F. Strauss III, and D. L. George. 1994. Enhanced translation: a novel mechanism of mdm2 oncogene overexpression identified in human tumor cells. *Oncogene* **9**:2745–2750.
42. Lane, D. P., C. W. Stephen, C. A. Midgley, A. Sparks, T. R. Hupp, D. A. Daniels, R. Greaves, A. Reid, B. Vojtesek, and S. M. Picklesley. 1996. Epitope analysis of the murine p53 tumour suppressor protein. *Oncogene* **12**:2461–2466.
43. Leach, F. S., T. Tokino, P. Meltzer, M. Burrell, O. J. D., S. Smith, D. E. Hill, D. Sidransky, K. W. Kinzler, and B. Vogelstein. 1993. p53 mutation and MDM2 amplification in human soft tissue sarcomas. *Cancer Res.* **53**:2231–2234.
44. Levine, A. J. 1990. The p53 protein and its interactions with the oncogene products of the small DNA tumor viruses. *Virology* **177**:419–426.
45. Luna, R. M. D., D. S. Wagner, and G. Lozano. 1995. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378**:203–206.
46. Manfredi, J. J., and C. Prives. 1994. The transforming activity of simian virus 40 large tumor antigen. *Biochim. Biophys. Acta Rev. Cancer* **1198**:65–83.
47. Marechal, V., B. Elenbaas, J. Plette, J. Nicolas, and A. J. Levine. 1994. The ribosomal L5 protein is associated with MDM2 and MDM2-p53 complexes. *Mol. Cell. Biol.* **14**:7414–7420.
48. Martin, K., D. Trouche, C. Hagemeier, T. S. Sorensen, N. B. La Thangue, and T. Kouzarides. 1995. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* **375**:691–694.
49. Michalovitz, D., D. Eliyahu, and M. Oren. 1986. Overproduction of protein p53 contributes to simian virus 40-mediated transformation. *Mol. Cell. Biol.* **6**:3531–3536.
50. Michalovitz, D., O. Halevy, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62**:671–680.
51. Mietz, J. A., T. Unger, J. M. Huibregtse, and P. M. Howley. 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J.* **11**:5013–5020.
52. Momand, J., G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**:1237–1245.
53. Montano, X., and D. P. Lane. 1984. Monoclonal antibody to simian virus 40 small t. *J. Virol.* **51**:760–767.
54. Mosner, J., and W. Deppert. 1994. p53 and mdm2 are independently expressed during cellular proliferation. *Oncogene* **9**:3321–3328.
55. Mosner, J., T. Mummenbrauer, C. Bauer, G. Szakiel, F. Grosse, and W. Deppert. 1995. Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J.* **14**:4442–4449.
56. Mumby, M. C., and G. Walter. 1991. Protein phosphatases and DNA tumor viruses: transformation through the back door? *Cell. Regul.* **2**:589–598.
57. Oliner, J. D., K. W. Kinzler, P. S. Meltzer, D. L. George, and B. Vogelstein. 1992. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* **358**:80–83.
58. Oliner, J. D., J. A. Pietenpol, S. Thiagalingam, J. Gyuris, K. W. Kinzler, and B. Vogelstein. 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* **362**:857–860.
59. Olson, D. C., V. Marechal, J. Momand, J. Chen, C. Romocki, and A. J. Levine. 1993. Identification and characterization of multiple mdm-2 proteins and mdm-2-p53 protein complexes. *Oncogene* **8**:2353–2360.
60. Otto, A., and W. Deppert. 1993. Upregulation of mdm-2 expression in Meth A tumor cells tolerating wild-type p53. *Oncogene* **8**:2591–2603.
61. Picklesley, S. M., and D. P. Lane. 1993. The p53-mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53? *Bioessays* **15**:689–690.
62. Picklesley, S. M., B. Vojtesek, A. Sparks, and D. P. Lane. 1994. Immunochemical analysis of the interaction of p53 with MDM2: fine mapping of the MDM2 binding site on p53 using synthetic peptides. *Oncogene* **9**:2523–2529.
63. Picklesley, S. M., B. Vojtesek, A. Sparks, and D. P. Lane. 1994. Immunochemical analysis of the interaction of p53 and MDM2—fine mapping of the MDM2 binding site on p53, abstr. 118. *In* Program and Abstracts of the 7th p53 Workshop.
64. Prives, C. 1990. The replication functions of SV40 T antigen are regulated by phosphorylation. *Cell* **61**:735–738.
65. Prives, C., J. Bargonetti, P. N. Friedman, J. J. Manfredi, and E. H. Wang. 1991. Functional consequences of the interactions of the p53 tumor suppressor protein and SV40 large tumor antigen. *Cold Spring Harbor Symp. Quant. Biol.* **56**:227–235.
66. Reifemberger, G., L. Liu, K. Ichimura, E. Schmidt, and V. P. Collins. 1993. Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res.* **53**:2736–2739.
67. Scheidtmann, K. H., B. Echle, and G. Walter. 1982. Simian virus 40 large T antigen is phosphorylated at multiple sites clustered in two separate regions. *J. Virol.* **44**:116–133.
68. Scheidtmann, K. H. 1989. SV40 large T antigen induces a protein kinase responsible for phosphorylation of the cellular protein p53. *Curr. Top. Microbiol. Immunol.* **144**:85–88.
69. Scheidtmann, K. H., M. Buck, J. Schneider, D. Kalderon, E. Fanning, and A. E. Smith. 1991. Biochemical characterization of phosphorylation site mutants of simian virus 40 large T antigen: evidence for interaction between amino- and carboxy-terminal domains. *J. Virol.* **65**:1479–1490.
70. Segawa, K., A. Minowa, K. Sugawara, T. Takano, and F. Hanaoka. 1993. Abrogation of p53-mediated transactivation by SV40 large T antigen. *Oncogene* **8**:543–548.
71. Stürzbecher, H. W., M. Montenarh, and R. Henning. 1987. Enhanced protein phosphorylation in SV40-transformed and -infected cells. *Virology* **160**:445–455.
72. Tack, L. C., and J. H. Wright. 1992. Altered phosphorylation of free and bound forms of monkey p53 and simian virus 40 large T antigen during lytic infection. *J. Virol.* **66**:1312–1320.
73. Tiemann, F., and W. Deppert. 1994. immortalization of BALB/c mouse embryo fibroblasts alters SV40 large T antigen interactions with the tumor suppressor p53 and results in a reduced SV40 transformation-efficiency. *Oncogene* **9**:1907–1915.
74. Tiemann, F., J. Zerrahn, and W. Deppert. 1995. Cooperation of simian virus 40 large and small T antigens in metabolic stabilization of tumor suppressor p53 during cellular transformation. *J. Virol.* **69**:6115–6121.
75. Todaro, G. J., K. Habel, and H. Green. 1965. Antigenic and cultural properties of cells doubly transformed by polyoma virus and SV40. *Virology* **27**:179–185.
76. Wang, J. Y. J., E. S. Knudsen, and P. J. Welch. 1994. The retinoblastoma tumor suppressor protein. *Adv. Cancer Res.* **64**:25–86.
77. Wu, X., J. H. Bayle, D. Olson, and A. J. Levine. 1993. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* **7**:1126–1132.
78. Xiao, Z. X., J. Chen, A. J. Levine, N. Modjtahedi, J. Xing, W. R. Sellers, and D. M. Livingston. 1995. Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* **375**:694–698.
79. Zambetti, G. P., and A. J. Levine. 1993. A comparison of the biological activities of wild-type and mutant p53. *FASEB J.* **7**:855–865.
80. Zerrahn, J., and W. Deppert. 1993. Analysis of simian virus 40 small t antigen-induces progression of rat F111 cells minimally transformed by large T antigen. *J. Virol.* **67**:1555–1563.
81. Zerrahn, J., F. Tiemann, and W. Deppert. 1996. Simian virus 40 small T antigen activates the carboxy-terminal transforming p53-binding domain of large T antigen. *J. Virol.* **70**:6781–6789.