Abstract. We previously reported that protein kinase CK2 phosphorylates the human mdm2 (hdm2) protein at serine residue 269. This phosphorylation site is located in the central acidic, highly-conserved region of mdm2, which is responsible for the interaction with a number of proteins. Studying the influence of phosphorylation of mdm2 by CK2 upon interaction with some of these binding partners, we found that the retinoblastoma (Rb) protein bound more strongly to the unphosphorylated mdm2 than to its CK2-phosphorylated counterpart. An S269 phosphospecific antibody was generated, and reacted with a 60 kDa subpopulation of mdm2 in human cells. We created a mutant mdm2 with a serine to aspartic acid exchange at position 269, which was used to transfect mdm2 -/- cells. Cells transfected with the S269D mutant exhibited a different growth behavior than wild-type mdm2-expressing cells, which might be attributed to the altered Rb-mdm2 interaction.

Introduction

The mdm2 protein is a key regulator of the growth suppressor p53 (1). Mdm2 is transactivated by p53, and subsequently binds to p53 to inhibit its growth attenuating function, thus acting as a negative feedback regulator of p53 (2). Mdm2 acts in a double manner, first by blocking directly the transactivation domain of p53 and thus stopping the transactivation of target genes necessary for the induction of growth arrest or apoptosis. Moreover, mdm2 promotes the export of p53 from the nucleus to the cytosol and thus, by acting as an E3 ubiquitin ligase itself, initiates the proteasomal degradation of p53 (3). Acetylation of mdm2 by histone acetyltransferases inhibits the degradation of p53 (4). By simultaneous binding of p53 and mdm2 to the p14ARF protein, p53 is protected from degradation (5). In addition to p53-dependent functions of mdm2, many other activities have been discovered (6).

Mdm2 interacts with pRb (7) as well as with E2F1/DP1 (8), and mdm2 overexpression increases E2F-mediated trans-activation. This effect might either result from an interference of the Rb-E2F interaction or a direct stimulation of the E2F-dependent transcription. The pRb-E2F interaction with mdm2 might thus contribute to the p53-independent oncogenic properties of mdm2. A carboxyterminal region of mdm2 is known to bind to the mdm2-related protein mdmx (9). The central acidic region, which includes the CK2 phosphorylation site, binds to the acetylase p300 (10), the ribosomal protein L5 (11) and p14ARF (12). Remarkably, with this particular domain, mdm2 also interacts with three growth suppressor proteins, namely the retinoblastoma protein (Rb) (13), MTBP (14) and PML (15).

Mdm2 is a highly-phosphorylated protein (16) and at least some of its activities are regulated by phosphorylation and dephosphorylation. This has been shown for the phosphorylation of mdm2 at S17 by double-stranded DNA-dependent protein kinase (dsDNA PK) (17), S166 and S186 by Akt kinase (18), T216 by cdk2/cyclin A (19), S395 by ATM kinase (20, 21) and Y394 by c-abl (22). Recently, we identified another phosphorylation site of human mdm2, namely serine 269 in the central acidic region of mdm2, which is phosphorylated by protein kinase CK2. This serine residue has meanwhile been shown to be an in vivo target of CK2 by ourselves and the Meek group for the murine homologue (23). In the present study, we analyzed the functional relevance of phosphorylation at serine 269 for activities of mdm2. In pull-down experiments, we found that the phosphorylation at serine 269 attenuates the interaction of the Rb protein with mdm2. Moreover, we found differences in the growth of cells transfected with wild-type mdm2 or the corresponding S269D mutant, indicating that the phosphorylation of mdm2 by CK2 influences the growth properties of cells.

Materials and methods

Mammalian cells and transfection protocols. Saos tsp53 S138V cells derived from the p53 negative osteosarcoma line Saos-2 that have been stably transfected with the temperature-sensitive p53 mutant S138V (kindly provided by Dr K. Roemer, Homburg, Germany). Cells were maintained at 37°C in DMEM supplemented with 10% fetal calf serum in an atmosphere enriched with 5% CO2. Cells grown at 37°C express mutant p53. Upon shift to 31°C for at least 5 h, the...
conformation of p53 changes into wild-type (24). Cells are then committed to apoptosis 48 h after shift to 31˚C.

174-1 cells are double negative p53−/−/mdm2−/− mouse embryo fibroblasts (a kind gift of G. Lozano) (25). They were grown under the same conditions as Saos tsp53 S138V cells. Cells were transfected with different mdm2 constructs using Superfect® (Qiagen) according to the manufacturer’s instructions. Selection of the transfected cells was achieved by the application of hygromycin B (Invitrogen; 500 µg/ml medium) where negative cells died 48 h after addition of the antibiotic.

**MTT assay.** Cell proliferation and viability was determined with a colorimetric MTT-based assay [3-(4,5-dimethyl-thiazol-2-yl)] 2,5-diphenyl tetrazolium bromide, Sigma-Aldrich, Deisenhofen, Germany). The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. Cells were grown in 96-well plates in a final volume of 100 µl culture medium. The MTT labeling reagent (10 µl) (5 mg/ml PBS) was added to each well. The enzymatic reaction was allowed to take place for at least 1 h at 37˚C in a humidified atmosphere. The formation of purple crystals was monitored in a light microscope. Crystals were solubilized by adding 100 µl of a solubilization solution (10% w/v SDS, 0.01 M HCl) for at least 4 h or overnight at 37˚C until the crystals have been completely dissolved. The spectrophotometrical absorbance of the purple-blue formazan dye was determined in an ELISA reader at a wavelength of 595 nm.

**Expression plasmids.** The bacterial expression plasmid for human mdm2, pRSETA-mdm2, was described elsewhere (26). S269A and S269D mutants of human mdm2 were produced in an overlap extension PCR by using primers where the codon 269 for serine, TCA, was exchanged to GCA for aspartic acid, respectively. The coding sequences of wild-type and mutant mdm2 were cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) using restriction sites Nhe1/Kpn1. The plasmid provides a hygromycin B resistance. The introduction of point mutations was made against amino acids 273-296 of human ribosomal L5 protein. The GST tag antibody is commercially available from Amersham-Pharmacia.

**Expression and purification of recombinant proteins.** The cDNA for wild-type mdm2 was cloned into the bacterial expression vector pRSET A (Invitrogen), which allows the expression of proteins under the control of an IPTG (isopropylthiogalactoside) inducible T7 polymerase promoter. Plasmids were transformed into E. coli BL21 (DE3), and bacteria were grown at 37˚C to an O.D. 490nm of 0.5. Expression of the proteins was induced at 30˚C for 4 h by the addition of IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation at 6000 x g for 15 min. The bacterial pellet from a 1-liter culture was resuspended in 15 ml of denaturing extraction buffer (6 M guanidinium hydrochloride, 50 mM NaH2PO4, pH 8.0). Cells were lysed by stirring for 1 h at room temperature. Cell debris was removed by centrifugation (15,000 x g, 30 min, 4˚C) and the lysate was incubated with Ni2+-NTA agarose (Qiagen; 1 ml packed agrose/l culture volume) for 30 min at room temperature under gentle shaking. The matrix was washed with the extraction buffer until the O.D. 280nm was < 0.01. In the next washing step, 5 mM imidazole was added to the extraction buffer. Bound mdm2 was eluted with the extraction buffer containing 100 mM imidazole. The purified fraction was dialyzed against 50 mM Tris/HCl, pH 7.5, 150 mM KCl and 5 mM MgCl2.

The GST-fusion proteins were also expressed in E. coli BL21 (DE3). The expression of the proteins was induced for 2 h at 37˚C. The purification procedure differed for the diverse fusion proteins. The GST-L5 protein was affinity-purified with glutathione-sepharose according to the standard protocol recommended by the manufacturer (Amersham-Pharmacia). For purification of the Rb fragment, the method suggested by Kaelin et al (27) using 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP40 as an extraction buffer produced the best results. After removing non-bound proteins, the fusion proteins were left on the resin and used for GST pull-down assays. Proteins were analyzed by SDS polyacrylamide gel electrophoresis and Western blot analysis according to standard procedures.

**In vitro translation of mdm2 and cleavage assay.** Human wild-type mdm2, S269A or S269D mutant protein were in vitro translated from the pcDNA3.1 constructs in the presence of [35S]-methionine according to the manufacturer’s recommendations (Promega).

Cytosol of apoptotic cells were prepared by Chen et al (28). Briefly, Saos tsp53 S138V cells grown either at 37˚C for control purposes or at 31˚C for 48 h were harvested and then resuspended in 3 volumes of cytosol extraction buffer (10 mM HEPES/KOH, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, and Complete™ protease inhibitor cocktail from Roche Diagnostics, Mannheim, Germany). The mixture was incubated on ice for 15 min and then cells were disrupted by sonification. Nuclei and cell debris were removed by centrifugation at 15,000 x g for 10 min at room temperature. The supernatant was stored at -80˚C.

To check the significance of the phosphorylation of mdm2 by CK2 for the proteolytic cleavage by apoptotic caspases 5 µl
of the above described in vitro translation products were mixed with 20 μl of the cytosol and incubated for 1 h at 37°C. The reaction was stopped by the addition of sample buffer (130 mM Tris-HCl, pH 6.8, 0.02% bromophenol blue, 10% mercaptoethanol, 20% glycerol, and 4% SDS) and the samples were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

**GST pull-down assay.** To study the possible influence of the S269 phosphorylation on the interaction of selected binding partners 10 μl of the above described in vitro-translated mdm2 proteins were mixed with 20 μg of immobilized GST-RbÇ8 or GST-L5-fusion protein in a total volume of 200 μl binding buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40 for L5 protein, 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% NP40, 2 mM DTT, 0.05% bovine serum albumin BSA, and 5% glycerol for Rb) (13). Binding was allowed by gentle shaking for 2 h at 4°C. The resin with the bound proteins was washed 3 times with 1 ml of the corresponding binding buffer. Proteins were eluted with 30 μl of SDS sample buffer and analyzed by SDS polyacrylamide gel electrophoresis and Western blot analysis or autoradiography.

**Binding studies with homopolyribonucleotides.** Binding studies with homopolyribonucleotides were done essentially as described by Elenbaas et al (11). The in vitro-translated mdm2 proteins (15 μl) (wt, S269A or S269D mutant) were incubated with 20 μg nucleic acid immobilized on agarose [homo-poly (A)-ribonucleotide or homo-poly (G)-ribonucleotide; Sigma-Aldrich] in a total volume of 200 μl RNA-binding buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 0.1% NP40, 50 μM ZnCl2, 2% glycerol, and 1 mM DTT) for 2 h at 4°C. Resins were washed 3 times with 1 ml of RNA binding buffer and boiled in SDS sample buffer. The protein was separated by SDS polyacrylamide gel electrophoresis and visualized by autoradiography.

**In vitro phosphorylation.** Recombinant protein kinase CK2 was expressed and purified as described elsewhere (29). Recombinant bacterially-expressed mdm2 proteins (1 μg) were mixed with CK2 in 20 μl kinase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 10 mM ATP). The kinase reaction was performed for 30 min at 37°C. The reaction was stopped by the addition of 10 μl SDS sample buffer. After SDS gel electrophoresis and transfer to a PVDF membrane, phosphorylation of mdm2 at S269 was detected with the phosphospecific antibody AK304.

**In vitro-translated mdm2 was immunoprecipitated using SMPI4.** The immunocomplex was washed 3 times with NET buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.02% NaN3, 5% sucrose, and 1% NP40) and twice with kinase buffer. Phosphorylation was performed under the same experimental conditions as described for the bacterially-expressed protein.

**SDS polyacrylamide gel electrophoresis and Western blot analysis.** Proteins were analyzed by SDS gel electrophoresis according to the procedure of Laemmli (30). For Western blot analysis, proteins were transferred to a PVDF membrane by tank blotting with 20 mM Tris/HCl, pH 8.7, 150 mM glycine as transfer buffer. Membranes were blocked in PBS with 0.1% Tween-20 and 5% dry milk for 1 h at room temperature. The membrane was incubated with the primary antibody (usually in a dilution of 1:1000, unless otherwise stated) in PBS-Tween-20 with 1% dry milk for another hour. The membrane was then washed 3 times with PBS-Tween-20 before incubating with the peroxidase-coupled secondary antibody in a dilution of 1:30,000 in PBS-Tween-20 with 1% dry milk. Signals were developed and visualized by the Lumilight system of Roche Diagnostics.

For autoradiography of [35S]-methionine labeled proteins, SDS polyacrylamide gels were soaked in a solution of 30% methanol and 10% acetic acid for 30 min at room temperature. Subsequently, the gel was treated with 1 M sodium salicylate, pH 5.0-7.0, for another 30 min for enhancement (31). The gel was dried and exposed to film at -80°C.

**RT-PCR for the detection of mdm2 mRNA in 174-1 cells.** 174-1 cells stably transfected with mdm2 constructs were used to isolate mRNA using the Quickprep® Micro mRNA purification kit of Amersham-Pharmacia according to the manufacturer's protocol. The first strand cDNA synthesis was performed with SuperScript® II reverse transcriptase (RT) and oligo dT primer as suggested by Gibco BRL Life Technologies (Eggenstein, Germany). cDNA was applied to a polymerase chain reaction (PCR) with amplification primers for mdm2 (5’ primer, 5’-AGAGAGAGTGCAATACCAAC 3’; 3’ primer, 5’-AGAGACCTAGGGGAAGAA 3’). Mdm2 was amplified in 40 cycles with Taq polymerase (Amersham-Pharmacia) using denaturation at 94°C for 15 sec, annealing at 53°C for 45 sec and extension at 72°C for 1 min. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide.

**Results**

**Identification of a subpopulation of mdm2 phosphorylated at residue S269 in vivo with a phosphospecific antibody.** We have previously shown that mdm2 expressed in insect cells is phosphorylated in the central region at serine 269 (26). To analyze the presence of mdm2 phosphorylated at serine 269 in mammalian cells, we used Saos tsp53 S138V cells. At 37°C, these cells express mutant p53, which allows rapid proliferation. Upon shift to 31°C, the conformation of p53 is changed to wild-type leading to growth arrest and finally to apoptosis. One of the wild-type functions of p53 is the transactivation of target genes among them its own negative regulator, mdm2. As shown in Fig. 1, mdm2 protein could be detected by Western blot analysis from 5 h after shift to at least 48 h. In addition to the 90 kDa variant of mdm2, a 60 kDa isofrom and perhaps a 54 kDa isofrom appeared after 24 h, representing the largest part of the mdm2 populations after 48 h. To study the phosphorylation of the mdm2 products, we generated an antibody against the phosphorylated CK2 epitope. The antibody was tested with recombinant mdm2, which was left untreated or phosphorylated with CK2 in Western blot analysis. As shown in Fig. 2, the antibody only recognized the phosphorylated form of recombinant mdm2 (lane b) and completely ignored the unphosphorylated
variant (lane a). Thus, having shown that the antibody exclusively recognizes mdm2, which is phosphorylated at serine residue 269, we analyzed a Saos tsp53 S138V extract with this phosphospecific antibody. Fig. 2 shows the result of the analysis. Saos tsp53 S138V extracts were analyzed either with the commercial antibody SMP14 (lane c) or the phosphospecific antibody (lane d). Whereas SMP14 recognized at least two forms of approximately 90 and 60 kDa, the phosphospecific antibody only recognized the 60 kDa isoform. Thus, we have shown that serine 269 of mdm2 is also phosphorylated in vivo, and that this phosphorylation appears at the 60 kDa variant of mdm2.

Influence of the phosphorylation of mdm2 at serine 269 on the apoptotic cleavage of mdm2. In certain cell lines, mdm2 is cleaved at aspartic acid 361 by a caspase-3-like enzyme during apoptosis, thus producing a 60 kDa variant of mdm2, whereas in some tumor cell lines this isoform is constitutively expressed. Because phosphorylation by CK2 has been described to be involved in preceding the apoptotic cleavage of some proteins, and because we also detected a 60 kDa form with the phosphospecific antibody, we wondered whether the phosphorylation at serine 269 might be a prerequisite for the cleavage of mdm2 by apoptotic caspases. We therefore used mdm2 in its wild-type, and S269A and S269D mutant forms. All were in vitro translated in the presence of [35S]-methionine. Equal amounts of the in vitro-translated proteins were incubated with the cytosolic extract which derived from Saos tsp53 S138V cells grown at 37˚C for 48 h. Cytosolic proteins of these cells and those of 37˚C control cells were extracted according to the protocol of Chen et al (28) to get apoptotic cell extracts. In vitro-translated mdm2 in its wild-type and mutant forms were mixed with the cytosolic extracts. After 1 h at room temperature, the reaction was stopped by adding denaturing sample buffer. Proteins were analyzed on an SDS polyacrylamide gel followed by fluorography. As shown in Fig. 3A, the mutant forms as well as the wild-type form of mdm2 were cleaved into the 60 kDa form (lanes c). An untreated mdm2 (lanes a), and mdm2 protein (lanes b), which was incubated with cytosolic extract from non-apoptotic cells as control, are shown. To be sure that the S269D mutant mimics phosphorylation, we repeated the experiment with in vitro-translated mdm2, which was or was not phosphorylated by protein kinase CK2. As shown in Fig. 3B, there was no difference between the cleavage of the phosphorylated (lane c) or non-phosphorylated product (lane b). Control with an untreated in vitro-translated mdm2 is shown in lane a.
Influence of the phosphorylation of mdm2 at serine 269 on the interaction with known binding partners. Besides p53, mdm2 binds to a variety of other cellular factors that were summarized by Momand et al (2). In addition, mdm2 is an RNA-binding protein (11) with two RNA-binding regions, one in the C-terminal RING finger domain and another in the central acidic region that has been shown to exclusively bind homopolyribonucleotides of the poly (G)-type. To study a possible influence of the phosphorylation by CK2, we translated mdm2 wt (lanes a), S269A (lanes b) or S269D (lanes c) mutant mdm2 proteins. After incubation, proteins were separated on a 10% polyacrylamide gel. The samples were washed to remove non-specifically bound proteins and separated on a 10% polyacrylamide gel. The binding partners were visualized by fluorography (for mdm2) or Western blot analysis with an Rb- or L5-specific polyclonal rabbit antiserum, respectively. (A) Binding of GST-L5 to mdm2, and (B) binding of GST-Rb to mdm2. Lanes a show the control with the respective GST-fusion protein alone.

Influence of the phosphorylation of mdm2 by CK2 on the growth of mammalian cells. We addressed the question of whether CK2 phosphorylation of mdm2 might have an effect on the proliferation of mammalian cells. For our studies, we used mouse embryo fibroblasts double negative for p53 and mdm2 (25). These cells were transfected with wild-type mdm2 or the phosphorylation mutants S269A and S269D.
shown in Fig. 6B. For the control 174-1 cells, we observed an
growth rate was determined by an MTT assay, which detects
cell density, and cell growth was recorded over 6 days. The
mdm2 in detail. All three cell lines were cultured at a similar
cells and those transfected with wild-type or the D-mutant of
mutant (lane A). Therefore, we only analyzed the parental
S269D mutant (lane D), but only a faint signal for the alanine
found in all three cases, but at different levels; lane (+) shows
primer. The PCR products are shown in Fig. 6A. As expected,
mRNA and performed an RT-PCR with mdm2-specific
embryo fibroblasts. 174-1 MEF cells were stably transfected with
mdm2, as well as in human and mouse mdmx protein,
resulting in the stabilization and accumulation of p53 (20).
To show that the cells expressed mdm2, we isolated the
mRNA and performed an RT-PCR with mdm2-specific
primers. The PCR products are shown in Fig. 6A. As expected,
there was no signal for mdm2 from the parental 174-1 cells
(lane c). The expression of recombinant DNA transcripts was
found in all three cases, but at different levels; lane (+) shows
a positive control with an mdm2 plasmid as a template. We
obtained a strong signal for the wild-type mdm2 (lane wt) and
S269D mutant (lane D), but only a faint signal for the alanine
mutant (lane A). Therefore, we only analyzed the parental
cells and those transfected with wild-type or the D-mutant of
mdm2 in detail. All three cell lines were cultured at a similar
cell density, and cell growth was recorded over 6 days. The
growth rate was determined by an MTT assay, which detects
metabolically active cells. Growth curves for the cells are shown in Fig. 6B. For the control 174-1 cells, we observed an
increase in cell number over the entire period. Cells expressing
wild-type mdm2 showed a stimulated growth. In contrast,
cells expressing the phosphorylation mutant S269D showed a
similar growth behavior to the parental cells. Thus, we have shown that phosphorylation at serine 269 has a significant
influence on the growth of cells.

Discussion

Mdm2 exists in different variants in the cell, which represent
either alternative splice products or differently-modified
polypeptides (16). All of these different mdm2 polypeptides
have been shown to be highly phosphorylated in vivo. Most
of the phosphorylation sites were identified in the amino-
terminal and central region of mdm2 (33). There is ample
evidence that some functions of mdm2 are regulated by
phosphorylation at distinct residues. Serine at residue 17 is
phosphorylated by dsDNA PK in response to DNA strand
breaks. The phosphorylation inhibits binding to p53, and thus
releases p53 to resume its growth suppressor functions (17).
The Akt/PKB kinase, a downstream target of the PI3 kinase,
phosphorylates mdm2 at residues 166 and 186. This phos-
phorylation promotes the translocation of mdm2 from the
cytosol to the nucleus, resulting in an enhanced p53 degradation
and abrogation of its transcriptional activities (18). Moreover,
the interaction of mdm2 with p300 is enhanced, whereas the
interaction with the Arf protein is inhibited (34). Threonine
216 is a target of the cyclin dependent kinase cdk2/cyclin A
and phosphorylation at this residue attenuates p53 binding
and increases the interaction with Arf (19). Tyrosine 394 and
serine 395 are the most carboxyterminal-identified phos-
phorylation sites. Under normal conditions, especially in
response to DNA damage, tyrosine 394 is phosphorylated by
the non-receptor tyrosine kinase c-abl. This phosphorylation
leads to a stabilization of p53 (22), which is normally degraded
under the influence of mdm2 (3). By replacing tyrosine 394
of mdm2 with phenylalanine, p53 is degraded faster, and its
transcriptional and apoptotic activities are inhibited. Serine
395 of mdm2 is a target of the radiation-sensitive kinase ATM
resulting in the stabilization and accumulation of p53 (20).

We have previously demonstrated that human mdm2 is
phosphorylated by protein kinase CK2 at position serine 269
(26). This serine residue is part of the highly-conserved
region II and is perfectly conserved in mouse and Xenopus
mdm2, as well as in human and mouse mdmx protein,
including a general role in the regulation of mdm2 (1,35).
Region II encompasses Ser242 to Pro331, and thus contains
the central acidic domain of the protein (Asp243 to Asp301).
We have now demonstrated that residue Ser269 is also
phosphorylated in vivo using a phosphospecific antibody.
This phosphorylation could only be detected in a subpopulation
of mdm2. Either the site is masked by another post-translational
modification in the full-length protein or the phosphorylation
event immediately follows or happens after the fragmentation
of mdm2. Mdm2 is cleaved by caspase 3 (CPP32) during
p53-mediated apoptosis between Asp361 and Cys362, thus
producing a 60 kDa mdm2 fragment (28,36). Pochampally et al
observed that before the activation of apoptotic caspases,
another caspase is activated that specifically cleaves mdm2.
The resulting 60 kDa fragment does not promote p53
degradation. Rather, it acts in a dominant negative fashion to protect p53 from degradation by full-length mdm2. In addition, this 60 kDa isoform of mdm2 was found in non-apoptotic tumor cells where it is not only the cleavage product of caspase 3, but also another protease (38). Most of the p53 in those cells is bound to p60, suggesting that it functions in the regulation of p53. The high level of p60 in tumor cells (39,40) suggests that production of p60 may be an integral part of the mdm2 pathway. Although, there are several examples that phosphorylation by CK2 precedes the cleavage of some proteins (41-43), we presented data that the phosphorylation at Ser269 is not a prerequisite for the formation of the 60 kDa form of mdm2.

The central acidic domain has been shown to interact with a number of cellular factors such as the ribosomal protein L5 (35), MTBP growth suppressor (14), p14ARF (5,12,44), Rb protein (13,45), TATA-binding protein (46), and homopolyribonucleotides (11). Binding of homopolyribonucleotides is an initial test for RNA-binding proteins and presumably represents a conserved function in a post-transcriptional regulation process (47). In the experiments presented, we never found a binding of mdm2 to poly (A)-ribonucleotides. However, mdm2 strongly bound to poly (G)-ribonucleotide. The alanine mutant bound best, which might indicate an impaired RNA-binding behavior for CK2-phosphorylated mdm2.

It was already shown that the Rb protein interacts with mdm2 when bound to p53 (13). The three proteins form a trimeric complex, thus inhibiting the p53-mediated transcription. Binding of Rb blocks the ability of mdm2 to initiate the degradation of p53 and mdm2 fails to prevent p53-mediated apoptosis. A critical feature for the growth-suppressing activity of Rb is the Rb-E2F network. Rb binds to the S-phase promoting factors E2F/DP1 (48), and by this interaction the transcription of genes involved in S-phase progression is repressed. Mdm2 binds to the same domain on the polypeptide chain of Rb (45) and displaces the transcription factor E2F. Thus, mdm2 is able to trigger S-phase progression by two different mechanisms: i) by directly binding to E2F and enhancing its transcriptional activity; and ii) through inhibition of Rb-binding to E2F by competition. In our experiment, the alanine mutant, which mimics non-phosphorylated mdm2, bound better to the Rb protein than the wild-type and aspartic acid mutant of mdm2.

The interaction between mdm2 and the growth suppressor Rb could also explain the observations made in cells transfected with mdm2 wild-type and mutants. It was expected that the Rb-mdm2 interaction is attenuated upon phosphorylation by CK2 (represented by the D mutant), and the Rb growth suppressor protein is released. Rb would then interact with E2F/DP1 transcription factors and thus repress the transcription of genes important for S-phase progression. As suggested, cells transfected with the S269D mutant showed a slower growth than those transfected with wild-type mdm2. As we used mdm2/p53 double knockout cells for the transfection experiments, the interference of mdm2 with p53 could not account for this finding. By analyzing the corresponding murine mdm2 mutants in vivo, Hjerrild et al (23) found that the S267A mutant directly degrades the direction of p53 at a slightly reduced rate.

Recently, CK2 has been described to be involved in enhancing the degradation of β-catenin, a key component of the wnt signal transduction pathway (49). On the other hand, it was also observed that CK2 phosphorylates and activates dishevelled (50) and β-catenin (51), and thus stimulates the signal transduction by the wnt pathway. Therefore, the role of CK2 in proliferation seems to be conflicting in some aspects, and the same might be true for the mdm2 protein. Moreover, the 60 kDa fragment, but not the full-length protein, seems to be the physiological substrate for CK2, and the phosphorylation of mdm2 by CK2 might have additional unexpected functions.

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References


