Cyclin A-CDK phosphorylation regulates MDM2 protein interactions

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Running title: Cyclin A-CDK complexes phosphorylate MDM2 at T216
Summary

The product of the MDM2 gene interacts with and regulates a number of proteins, in particular the tumor suppressor p53. MDM2 protein is likely to be extensively modified in vivo and such modification may regulate its functions in cells. We identified a potential cyclin-dependent kinase (CDK) site in murine MDM2, and found the protein to be efficiently phosphorylated in vitro by cyclin A containing complexes (cyclin A-CDK2 and cyclin A-CDK1) but MDM2 was either weakly or not phosphorylated by other cyclin-containing complexes. Moreover, a peptide containing a putative MDM2 cyclin recognition motif specifically inhibited phosphorylation by cyclin A-CDK2. The site of cyclin A-CDK2 phosphorylation was identified as T216 by 2-dimensional phosphopeptide mapping and mutational analysis. Phosphorylation of MDM2 at T216 both weakens its interaction with p53 and modestly augments its binding to p19ARF. Interestingly, an MDM2-specific monoclonal antibody, Mab SMP14, cannot recognize MDM2 phosphorylated at T216. Changes in SMP14 reactivity of MDM2 in staged cell extracts indicate that phosphorylation of MDM2 at T216 in vivo is most prevalent at the onset of S phase when cyclin A first becomes detectable.
Introduction

The mdm2 gene was originally cloned from a spontaneously transformed mouse cell line 3T3DM (1). Its potential pro-oncogenic activity was supported by the observation that amplification or over-expression of MDM2 promotes tumorigenesis in NIH 3T3 or Rat2 cell lines (2). In cooperation with ras, MDM2 over-expression can also promote transformation of primary rodent fibroblasts (3). The finding that MDM2 is amplified in ~30% of human sarcomas (4) further suggests that it is a proto-oncogene that promotes cell proliferation.

The murine MDM2 protein contains several conserved functional regions. Its N-terminus encodes a domain that interacts with p53 (residues 26-108) (5-8). Adjacent to nuclear localization (NLS; residues 178-182) (9) and nuclear export (NES; residues 183-195) (10) signal sequences is a central acidic domain (residues 211-299) that has been shown to modulate transcriptional activity (10,11) and to interact with proteins. The C-terminus of MDM2 contains a RING finger domain (residues 442-481) that is important for ubiquitin-mediated degradation or SUMO-mediated stabilization of p53 (12-14).

One important function of the MDM2 protein is to down-regulate p53 protein levels and p53 activity (reviewed in 15,16). In response to various cellular stresses, active p53 protein regulates numerous effectors that are involved in cell cycle arrest, apoptosis or other cellular processes (reviewed in 17). The interaction between the N-terminus of MDM2 and p53 proteins is important for inhibiting p53-mediated transactivation (18,19) as well as for targeting p53 for degradation (20-22). In turn, p53 protein can bind to the promoter of MDM2 and activate MDM2 transcription (23). This feed-back loop between MDM2 and p53 fine-tunes the cellular responses after p53 activation (24). Another tumor suppressor protein, p19ARF, can also bind to a
region of MDM2 spanning its central acidic domain (25-27, reviewed in 28). In some cases, p19\textsuperscript{ARF} renders MDM2 inactive in regulating p53 protein by translocating MDM2 into the nucleolus (29,30) and inhibiting the ubiquitin ligase activity of MDM2 (31). The importance of MDM2 in p53 down-regulation has also been shown by genetic analysis. \textit{Mdm2}-null mouse embryos die by day 5 of gestation while mice null for both \textit{mdm2} and \textit{p53} genes are viable (32,33). The rescue of embryonic lethality of \textit{mdm2}-null mice by \textit{p53} deletion implies that the MDM2 protein is essential for p53 regulation during early embryogenesis.

In addition to its interaction with p53 protein, MDM2 possesses other activities that promote cell proliferation. It was reported to interact with and stimulate the S-phase promoting factor E2F1 (34,35). MDM2 also binds to the retinoblastoma (pRb) tumor suppressor protein \textit{in vitro} and inhibits the growth regulatory function of pRb (36). In TGF-\(\beta\)-resistant human breast cancer cells, MDM2 over-expression is correlated with failure to produce the growth inhibitory form of pRb and to reduce E2F1 protein levels after TGF-\(\beta\) treatment (37). This regulation is independent of the p53 status of the cells.

Genetic studies of transgenic mice with targeted MDM2 over-expression in the mammary gland also elucidate the effect of MDM2 in promoting cell cycle progression, especially entry into S-phase (38,39). MDM2 transgenic mice have abnormal mammary gland development and a high incidence of mammary tumors. Over-production of MDM2 protein uncouples S-phase from mitosis, which leads to the production of mammary epithelial cells that are polyploid and have nuclear abnormalities. P53 deletion has no effect on this phenotype; neither does E2F1 deletion or over-expression. The uncoupling of S-phase has been correlated with elevated cyclin A but not cyclin E or cyclin D mRNA levels in MDM2 over-expressing cells (38). MDM2 expression was reported to lead to activation the cyclin A promoter (10). This may be related to
interactions of MDM2 with components of the basal transcription factor TFIID: the MDM2 C-terminal RING finger domain interacts with TAFII250 and the region close to the acidic domain interacts with TBP (10,11,40,41).

Cell cycle progression depends upon the formation of active cyclin-CDK complexes that are regulated by the synthesis and degradation of cyclins, their state of phosphorylation, and their association with inhibitory subunits (reviewed in 42-45). Cyclin A, which forms complexes with CDK2 at the beginning of S-phase and CDK1 at the beginning of M-phase, is required for entry into S-phase, passage through G2 and mitosis (46-49). The substrates of cyclin A-CDK2 include a number of proteins, including the p53 protein(50).

MDM2 is likely to be extensively regulated by phosphorylation. More than one third of the amino acids on MDM2 protein are either serine or threonine residues, and MDM2 protein is phosphorylated at multiple sites in vivo, especially in the N-terminus and the central acidic domain (51). It has been reported that ATM kinase(52), DNA-dependent protein kinase (DNA-PK)(53) and casein kinase 2 (CK2) (54,55) can phosphorylate MDM2 proteins and modulate MDM2 functions. Murine MDM2 has at least one potential site for CDK phosphorylation. We report here that cyclin A-CDK complexes are unique in their ability to efficiently phosphorylate MDM2 and that this phosphorylation affects the interactions of MDM2 with proteins.
Experimental Procedures

Purification of Proteins

A construct expressing GST-MDM2 was kindly provided by M. Oren (Weizmann Institute). Mutant GST-MDM2 T216A and T384A constructs were generated from wild-type GST-MDM2 using the Stratagene QuickChange® Mutagenesis kit. Bacterially expressed GST-MDM2 proteins were purified from Gluthione-Sepharose 4B columns (GIBCO-BRL). Complexes containing cyclin E and CDK2 (300 ng cyclin E and 600 ng CDK2 in 10 µl), cyclin A and CDK2 (200 ng cyclin A and 300 ng CDK2 in 10 µl), cyclin B and CDK1 (12 ng cyclin B and 25 ng CDK1 in 10 µl), or cyclin A and CDK1 (10 ng cyclin A and 150 ng CDK1 in 10 µl) were purified from extracts of insect Sf9 cells that had been co-infected with these respective pairs of recombinant baculoviruses as previously described (50). A purified complex containing cyclin D1 and CDK4 was generously provided by Y. Taya (56). Murine p53 protein was isolated from extracts of baculovirus-infected insect cells by immunoaffinity purification over a PAb 421 column as previously described (57). A construct expressing His-tagged p19ARF-N-37 was kindly provided by the laboratory of C. Sherr and was purified from bacteria as described (58).

In vitro Kinase Assays

Kinase assay mixtures (30 µl) contained kinase buffer (50 mM Hepes [pH7.5], 10 mM MgCl2, 1 mM DTT, 100 µM ATP, and 4 µCi γ-[32P] ATP) and 100 ng purified GST-MDM2, pRb, or histone H1 proteins as substrates. Cyclin-CDK complexes were added into reaction mixtures, which were incubated at 30°C for 15 minutes, and then resolved on 10% SDS-
polyacrylamide gels. The gels were either silver-stained or stained with Coomassie Blue to detect protein levels before exposure to X-ray film. Peptides used in the cyclin recognition motif experiments were synthesized by Synpep.

**Two Dimensional Phosphopeptide Mapping**

$[^{32}\text{P}]$ labeled GST-MDM2 (1 µg) was phosphorylated by cyclin A-CDK2 as described above and then run on a 10% SDS-polyacrylamide gel and exposed to X-ray film. The phosphorylated GST-MDM2 full-length protein was then cut out and eluted from the gel slice in buffer containing 0.05M NH$_4$HCO$_3$ (pH 7.3), 0.5% β-mercaptoethanol and 0.1% SDS. The eluted proteins were acid-precipitated and oxidized as described (59), and then digested with 10 µl V8 protease (1 µg/µl) at 37°C for 16 hours in 50 µl buffer containing 0.05M NH$_4$HCO$_3$ (pH 7.3) (60). The proteolytically digested MDM2 was subjected to electrophoresis in pH 1.9 buffer in the first dimension and chromatography in Phospho-Buffer (N-butanol / pyridine / glacial acetic acid / H$_2$O :: 15/10/3/12) in the second dimension as described (59).

**ELISA Assay**

Baculovirus-expressed murine wild-type p53 protein (50 ng), or bacterially expressed p19$^\text{ARF}$-N-37 peptide (20 ng) were used to coat each well in a 96-well Pro-bind plate (Falcon) in 200 µl PBS for 2 hours in 4°C. The wells were then washed three times in PBS containing 0.05% Tween and then incubated in blocking buffer (PBS containing 0.05% Tween and 1% bovine serum albumin) for 1 hour at 4°C. Phosphorylated or unphosphorylated GST-MDM2 (15-180 ng) in 200 µl PBS was then added to each well and incubated for 1 hour at 4°C. Roscovitine (10 µM) was present in the PBS to prevent the kinase from phosphorylating the
coating protein. The wells were briefly washed three times with PBS containing 0.05% Tween, at which time anti-MDM2 monoclonal antibody, either a 1:1000 dilution of purified Mab SMP14 (200 µg/ml, Santa Cruz) or a 1:40 dilution of Mab 4B11 hybridoma supernatant (generously provided by A. Levine) in blocking buffer, was added and incubated for 1 hour at room temperature. After three washes, a 1:2000 dilution of monoclonal anti-mouse IgG antibody conjugated to alkaline phosphatase (SIGMA) in blocking buffer was added and incubated at room temperature for 30 minutes. After five washes, 10 mM PnPP (p-nitrophenol phosphate, SIGMA) in 100 mM AMPP (2-amino-2-methyl-1,3-propanediol, SIGMA) was added to the wells. Absorbance at 405 nm was measured at 10-minute intervals using a Bio-Rad Model 550 Microplate Reader. The amount of MDM2 bound to the coating protein was calculated from the change in absorbance at different time points. Each data point is an average of readings from triplicate wells.

Western Blot Analysis of Cell Extracts

Swiss 3T3 mouse fibroblast cells were maintained in DMEM with 10% fetal bovine serum (FBS). Log-phase cells were starved in DMEM with 0.5% FBS for 48 hours before being released into DMEM containing 10% FBS. Cell cultures (1 plate each) were collected at different time points and washed twice in ice-cold PBS. The cells were scraped, centrifuged at 1500 rpm in 4°C and then lysed in extraction buffer (PBS containing 2% SDS and 10 mM iodoacetamide). Extracts were then heated at 95°C for 10 minutes and sonicated for 10 minutes. Protein concentrations were determined by the BCA Assay (Pierce) and 150 µg of each extract was run on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Schleicher & Schuell). MDM2 protein was detected by Western blot analysis using anti-MDM2
antibodies, either Mab SMP14 (Santa Cruz) or Mab 3G5 hybridoma supernatant (a gift from Dr. A. Levine). The same blots were also probed with an anti-cyclin A polyclonal antibody H437 or an anti-cyclin B monoclonal antibody Ab-2 (both from Santa Cruz). Duplicate plates of cells at each time point were collected and processed for FACS analysis.

**GST Pull-down Assay**

GST-tagged proteins in kinase reaction mixtures were incubated with Gluthione Sepharose 4B beads (GIBCO-BRL) in 200 µl ice-cold TEGN washing buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 150 mM NaCl, 10 % glycerol, 0.5% NP40, and 0.5 mM sodium orthovanadate) for 1 hour at 4 °C. The beads were centrifuged at 2500 rpm, and then washed twice with TEGN buffer and blocked in 200 µl TEGN buffer containing 1 % BSA at 4 °C. Beads containing 100 ng GST-tagged protein were rocked with mouse p53 protein in 200 µl TEGN buffer at 4 °C for 1 hour, washed five times with the same buffer, and then separated on a 10% SDS-polyacrylamide gel. GST-MDM2 and mouse p53 protein were detected by Western blot analysis with an anti-MDM2 rabbit polyclonal antibody or a mixture of mouse p53 monoclonal antibodies: PAb 242, PAb 246 and PAb 248.
Results

GST-MDM2 is preferentially phosphorylated by cyclin A containing CDK complexes

Examination of the sequence of murine MDM2 protein indicated that there are two possible CDK sites in the protein at residues 216 (TPSH) and 384 (TPLS). In fact, T216-P217 is conserved between murine and human (T218-P219) MDM2. Using bacterially expressed full-length murine GST-MDM2 as the substrate, we performed in vitro kinase assays with increasing amounts of purified cyclin A-CDK2 complex (Figure 1). GST-MDM2 was phosphorylated by cyclin A-CDK2 in a dose-dependent fashion. At the highest level of kinase complex added, the mobility of MDM2 protein was completely shifted to a slower migrating form indicating that it had been efficiently phosphorylated (Figure 1, lane 6,7). To ensure that the phosphorylation observed was the result of cyclin A-CDK2 activity rather than a contaminating kinase we used a CDK2 inhibitor roscovitine (ID$_{50}$=700nM, specific for CDK1 and CDK2) (61,62). The incorporation of label from [$_{32}$P]ATP was substantially decreased by roscovitine with a concomitant increase in electrophoretic mobility of the MDM2 polypeptide when compared to the phosphorylated form (Figure 1, compare lanes 7 and 8).

We then investigated the abilities of other cyclin-CDK complexes to phosphorylate GST-MDM2. The activities of the complexes were normalized using either histone H1 or pRb as a positive control. Although the cyclin E-CDK2 complex has the same kinase component as cyclin A-CDK2, it only very weakly phosphorylated GST-MDM2 (Figure 2A, lane 6-9). Cyclin D1-CDK4 was also impaired in phosphorylating GST-MDM2 when compared to its ability to phosphorylate pRb protein (Figure 2B, lane 1,2 vs. lane 3,4). It is interesting as well that the cyclin B-CDK1 complex phosphorylated GST-MDM2 more modestly than did cyclin A-CDK2.
(Figure 2C, lane 5-8) even though, as expected, both kinases phosphorylated histone H1 to roughly the same extent. Since cyclin A has been shown to form a complex with CDK1 at the onset of mitosis (47), we then compared the abilities of purified cyclin A-CDK1 and cyclin B-CDK1 complexes to phosphorylate GST-MDM2 \textit{in vitro} (Figure 2D). Indeed, cyclin A-CDK1 phosphorylated GST-MDM2 and, like cyclin A-CDK2, did so more effectively than cyclin B-CDK1. Therefore, cyclin A containing CDK complexes are the most effective in phosphorylating MDM2 protein.

**Cyclin A-CDK2 phosphorylates MDM2 at T216**

To identify the CDK site(s) in MDM2, either T216 or T384 was mutated to alanine in full-length GST-MDM2, and wild-type and T216A or T384A MDM2 proteins were purified from bacteria and treated with cyclin A-CDK2 in mixtures containing $^{32}$PATP. We then performed 2-D mapping of phosphopeptides generated from V8 protease digestion of phosphorylated wild-type or mutant proteins. In the map of the phosphorylated wild-type protein, a predominant phosphopeptide was observed (Figure 3A, arrow). This spot was diminished when the CDK2 specific inhibitor roscovitine was added to the kinase reaction mixture (Figure 3B), supporting the possibility that it is specifically phosphorylated by cyclin A-CDK2. Importantly, this phosphopeptide was absent in the 2-D map of the T216A mutant (Figure 3C) but was present in the map of the T384A mutant (Figure 3D). The additional minor phosphopeptides that were in the 2-D maps are most likely the result of cryptic phosphorylation by the CDK complex since they are largely suppressed by roscovitine. We conclude that cyclin A-CDK2 phosphorylates predominantly T216 in murine MDM2.
MDM2 contains a cyclin recognition motif (CRM)

Phosphorylation of a number of cyclin dependent kinase substrates requires their interaction with cyclins mediated by sequences (CRMs) termed cyclin-CDK substrate recognition motifs (63-68). Located between the NLS and NES signals on murine MDM2 is a sequence containing RRSL (residues 181-184) which fits the consensus sequence ZRXL for CRMs in E2F, p107, p21, p27, pRb and poly A polymerase (65, 67, 69-72, reviewed in 73,74). It is possible that this sequence may be involved in CDK phosphorylation of MDM2. Peptides containing the potential MDM2 CRM sequence (RKRRRRLSFDP), scrambled CRM sequence (LRPKSFRDSRR) or the CDK phosphorylation site (SESTETPSHQDL) were synthesized and used as competitive inhibitors in the kinase reaction mixtures. The CRM peptide efficiently inhibited cyclin A-CDK2 phosphorylation of MDM2 (IC₅₀ < 500 nM) while the scrambled CRM peptide was much less inhibitory (Figure 4, lanes 4 and 5). In addition, the MDM2 CRM peptide specifically inhibited cyclin A-CDK2 phosphorylation of pRb, also shown to be CRM dependent, but not the phosphorylation of histone H1 which does not require cyclin recognition. It is thus possible that this region may mediate a transient but critical interaction between cyclin A and MDM2. Although we failed to detect a stable interaction between GST-MDM2 and cyclin A (alone or in complex with CDK2) in a GST pull-down assay (data not shown), the phosphorylation of pRb only requires a transient interaction between the pRb cyclin-CDK recognition motif and cyclin-CDK complexes (67). Interestingly as well, the MDM2 CDK site peptide was unable to compete with GST-MDM2 for cyclin A-CDK2 phosphorylation (Figure 4, lanes 9-11) even at concentrations as high as 60 µM (data not shown). This further supports the likelihood that the MDM2 CRM sequence is required for MDM2 to be phosphorylated at T216 by the kinase complex.
MDM2 monoclonal antibody SMP14 cannot recognize GST-MDM2 phosphorylated by cyclin A-CDK2 or cyclin A-CDK1 at T216

During an ELISA experiment intended as a control for the effect of phosphorylation of MDM2 on protein-protein interactions, we unexpectedly discovered that Mab SMP14, an MDM2 specific monoclonal antibody, displays reduced binding to cyclin A-CDK2 phosphorylated GST-MDM2 protein. This was surprising because this antibody was generated using a peptide spanning human MDM2 (HDM2) residues 154-167. Nevertheless, reactivity with this antibody was clearly decreased after murine MDM2 protein was phosphorylated by cyclin A-CDK2 (Figure 5A). To eliminate the possibility that the effect was caused by either ATP binding or the presence of the kinase complex, we substituted ATP with a non-hydrolysable ATP analog, AMP-PNP, which was inactive in the kinase reaction. Mab SMP14 reactivity in this case was the same as with the unphosphorylated protein, indicating that the loss of recognition by this antibody was caused by cyclin A-CDK2 phosphorylation of MDM2 protein. In a separate ELISA experiment, we also discovered that Mab SMP14 could not recognize the T216A mutant protein (Figure 5B). Thus, the SMP14 monoclonal antibody can differentiate between an un-phosphorylated and phosphorylated murine MDM2 epitope around T216. These results were confirmed in a Western blot experiment in which increasing phosphorylation of MDM2 by the cyclin A-CDK2 complex caused progressive loss of reactivity with SMP14 (Figure 5C, lane 2-4). Furthermore, after the phosphorylated GST-MDM2 was treated with calf intestine phosphatase, Mab SMP14 reactivity was fully restored (Figure 5C, lane 5). The amount of the GST-MDM2 protein was uniform in all reactions as demonstrated by Western blot with Mab 3G5, another monoclonal antibody that does not differentiate the phosphorylated forms of GST-
MDM2. Consistent with the *in vitro* kinase assay, Mab 3G5-recognized protein was slightly up-shifted when GST-MDM2 was phosphorylated by the higher concentration of cyclin A-CDK2 (Figure 5C, lane 4). Phosphorylation of GST-MDM2 by cyclin A-CDK1 gave a similar result (Figure 5D), indicating that this kinase complex also phosphorylates the T216 residue. Further supporting the conclusion that cyclin A uniquely determines the specificity of phosphorylation of T216 on MDM2, is the observation that there was little or no difference between Mab SMP14 and Mab 3G5 reactivity when cyclin B-CDK1 was used to phosphorylate GST-MDM2 (Figure 5E).

**MDM2 is phosphorylated at T216 *in vivo* at the G1/S transition**

The fact that Mab SMP14 reactivity is sensitive to phosphorylation of T216 could provide a means to evaluate the modification of this site *in vivo*. Since cyclin A was the critical component of the kinase complex, it was of interest to determine whether Mab SMP14 reactivity varies with the cell cycle. To test this, Swiss 3T3 fibroblast cultures were first serum-starved and then released into the cell cycle by addition of serum-containing medium. At different time points after release, cultures were examined for cell cycle distributions by FACS analysis (Table 1) or MDM2 protein levels by Western blot analysis (Figure 6A). By 14 hours after cells were released from serum starvation, they started to go into S phase, and the number of G1/S-phase cells peaked at 16 hours after release. By 20 hours the cells began to move into G2/M and at 24 hours, half of the released cells were in G2/M. At later time points, the cells are no longer well synchronized. A decrease of SMP14-reactive MDM2 protein (relative to Mab 3G5-reactive MDM2) was evident by 14 hours (Figure 6A, lane 3). Quantification of the results by densitometry showed that the difference was greatest at 16 hours after release (Figure 6B). In
fact at 16 hours the Mab 3G5-reactive MDM2 protein displayed slightly reduced gel mobility, consistent with our results with MDM2 protein phosphorylated in vitro by cyclin A-CDK2. The G1/S transition observed in the FACS analysis coincided with the first detection of cyclin A protein at 14 hours after release (Figure 6A, lane 3). The existence of a subpopulation of MDM2 protein at S phase transition that was less well recognized by SMP14 antibody suggests that MDM2 protein becomes phosphorylated at T216 when cyclin A-CDK2 is first available. Later in the cell cycle, SMP14 reactive MDM2 is restored while cyclin A levels continue to rise. The reduction in T216 phosphorylated MDM2 later in the cell cycle may be the result of other cellular events that are explored further in the Discussion.

**Effects of cyclin A-CDK2 phosphorylation on the interaction of MDM2 with p53 or p19ARF**

Phosphorylation of MDM2 by DNA-PK was previously reported to diminish its interaction with p53 (53). Although T216 is not within the p53 interaction region on MDM2, it is still possible that other regions of the protein can regulate this interaction. Therefore, a modified ELISA assay (53) was employed to analyze the interaction between MDM2 and p53 after phosphorylation by cyclin A-CDK2. Because Mab SMP14 could not recognize phosphorylated MDM2 and the epitope for 3G5 lies in the p53-MDM2 binding region, we used another antibody specific for the MDM2 C-terminus, Mab 4B11, as the primary antibody to detect p53-interacting MDM2 protein. The amount of p53-bound MDM2 was measured by $\Delta A_{405\text{nm}}$, reflecting the alkaline phosphatase activity conjugated to the secondary antibody. Based on results with BSA as a negative control, non-specific MDM2-protein interaction was below the level of detection. We compared GST-MDM2 which was either unphosphorylated or phosphorylated in vitro by cyclin A-CDK2. For the unphosphorylated control MDM2, kinase
reaction mixtures were substituted with non-hydrolysable AMP-PNP. Our data indicate that phosphorylation by cyclin A-CDK2 negatively affects MDM2’s interaction with p53 (Figure 7A-I). Although phosphorylation did not abolish the p53/MDM2 interaction, there was ~ 40% reduction in the binding of phosphorylated MDM2 when compared to mock-phosphorylated MDM2. Because cyclin A-CDK2 can phosphorylate cryptic sites on MDM2 protein, the effect observed could be due to phosphorylation at residues other than T216. To eliminate such a possibility, we incubated mutant MDM2 (T216A) protein in kinase reaction mixtures containing either ATP or AMP-PNP, and then measured the binding to p53 in the ELISA assay. In this case no difference in p53-mutant MDM2 interaction was observed with or without cyclin A-CDK2 phosphorylation (Figure 7A-II), thus demonstrating that the negative effect of MDM2 phosphorylation by cyclin A-CDK2 on the interaction between MDM2 and p53 is caused by phosphorylation at T216. To further validate these results we employed a GST-pull down protein interaction assay in which either unphosphorylated or phosphorylated GST-MDM2 protein was incubated with mouse p53 and then complexes were isolated on Gluthione Sepharose beads. GST protein was used as a negative control for non-specific binding (Figure 7B, lane 1). Consistent with the ELISA data, phosphorylated GST-MDM2 protein bound less well to p53 protein than did the unphosphorylated protein (Figure 7B, compare lanes 3 and 4 to lanes 6 and 7).

The p19ARF interacting region on MDM2 within its acidic domain between residues 210 and 304 is potentially close to the cyclin A-CDK2 phosphorylation site (27,58). We therefore also tested whether CDK phosphorylation may also modulate the interaction of MDM2 with p19ARF. Here we used the MDM2-interacting N-37 fragment of p19ARF protein, which was purified from bacteria. When tested by ELISA, contrary to its effect on p53-MDM2 interaction,
cyclin A-CDK2 phosphorylation reproducibly produced a modest stimulation of the interaction between MDM2 and p19\textsuperscript{ARF}-N-37 at lower concentrations of MDM2 (Figure 7C-I). In the control ELISA experiment using mutant MDM2 T216A protein, we observed no significant difference (Figure 7C-II). Taken together our data suggest the possibility that phosphorylation at T216 serves to regulate the interaction of MDM2 with other proteins.
Discussion

The MDM2 protein plays an important role in regulating cellular proliferation. It has been shown to interact with a number of key regulators of the cell cycle such as p53, E2F and pRb proteins. Although MDM2 contains numerous potential modification sites, there is limited knowledge about how MDM2 is modified and the effects of those modifications (52-55). Here we have demonstrated that MDM2 can be phosphorylated by cyclin A containing CDK complexes, and have mapped the site of phosphorylation to T216 in the acidic domain of MDM2 protein. Additionally, we discovered that MDM2 monoclonal antibody SMP14 can differentiate between cyclin A-CDK2 phosphorylated MDM2 and unphosphorylated MDM2. Using this antibody as a tool, we provide evidence that MDM2 is phosphorylated at T216 at the onset of S-phase.

The observation that MDM2 is selectively phosphorylated by cyclin A containing complexes also led to the identification of a cyclin recognition motif on MDM2. The MDM2 CRM resembles that of pRb in that a stable interaction between the MDM2 and cyclin A could not be detected (67). We were able to demonstrate its importance in the functional kinase assay, however, because a peptide containing the MDM2 CRM sequence was able to inhibit cyclin A-CDK2 phosphorylation of MDM2 at low concentrations. Since this peptide can also inhibit pRb phosphorylation by cyclin A-CDK2, it will be of interest to explore its effect on the phosphorylation of other CRM-containing proteins and whether it has any physiological effect in vivo, as has been shown for the CRM of p21 protein(75).

It was unexpected that phosphorylation of MDM2 at T216 by cyclin A-CDK2 causes loss of reactivity with the MDM2-specific monoclonal antibody Mab SMP14. Mab SMP14 was...
generated by using a peptide spanning amino acids 154-167 on human HDM2 protein (7,76).
Our experiments support the likelihood that this region on HDM2 is the recognition site for Mab
SMP14 since an N-terminally deleted HDM2 lacking residues 1-166 does not react with this
antibody (data not shown). The human Mab SMP14 epitope is not completely conserved on
murine MDM2, however, and in fact we also observed that murine MDM2 is much more weakly
recognized by Mab SMP14 than its human counterpart (data not shown). When a 12-residue
peptide spanning the CDK site (sequence shown in Figure 4) was tested for its ability to block
SMP14 recognition of MDM2 by ELISA assay, no significant inhibition was observed (data not
shown). It is possible that this peptide is not long enough or does not have the right conformation
to be recognized by SMP14. Alternately the SMP14 epitope may be located elsewhere in
MDM2, and its recognition by the monoclonal antibody is negatively affected by either
phosphorylation or substitution of T216. This is not the first example of a monoclonal antibody
that fails to recognize phosphorylated MDM2: ATM phosphorylated HDM2 is not recognized by
the monoclonal antibody Mab 2A10 (52).

The ability of SMP14 to discriminate between phosphorylated and unphosphorylated
MDM2 allowed us to examine possible variations in phosphorylation at T216 in vivo. By
examining the passage of Swiss 3T3 cells through the cell cycle after release from serum
starvation we found that there was a significant drop in Mab SMP14 reactivity as cells began to
accumulate in S phase at roughly the same period when levels of cyclin A protein became
detectable. However, we only observed significant phosphorylation on T216 when the cells
began to go into S-Phase. At 20 hours after serum-release when most of the cells are in S-phase
and some even going into M-phase, Mab SMP14 reactivity is regained indicating that most of the
MDM2 protein is no longer phosphorylated on T216 even though cyclin A protein levels
continue to increase. Although we do not yet understand the reason for this, it appears that T216 is phosphorylated at the onset of S-phase and then either de-phosphorylated by as yet unidentified phosphatase(s) when the cells pass through S-phase, or new molecules of MDM2 cannot be phosphorylated at this site. With respect to the former possibility, it is intriguing that another transcriptional target of p53, cyclin G, has been shown to interact with protein phosphatase 2A (PP2A) (77). We are currently examining whether cyclin G-bound PP2A plays a role in the dephosphorylation of MDM2 protein in the cell. Alternately, CDK inhibitors such as p21, another p53 transcription target, may be involved. P21 has been reported to bind to cyclin A-CDK2 when the cell passes S-phase(78-80). Therefore, despite the increasing cyclin A expression in the cell cycle, the cyclin A-CDK2 complex may no longer be active to phosphorylate the newly synthesized MDM2.

It would be interesting to investigate whether cyclin A, whose promoter is transcriptionally regulated by MDM2, is differentially regulated by phosphorylated MDM2 protein. TBP interacts with MDM2 at a region adjacent to the acidic domain. This interaction is implicated in the cyclin A promoter activation (10), as well as transcriptional repression of p53 responsive elements (11). Deletion analysis indicated that the TBP-MDM2 interaction domain is close to T216 (10,11). When we examined whether cyclin A-CDK2 phosphorylation of MDM2 affects its interaction with TBP, however, the small negative effect we observed was not statistically significant (data not shown). It is therefore difficult to predict what the effect of cyclin A-CDK2 phosphorylation would be at the transcriptional level. Further \textit{in vivo} experiments will be employed to address this question.

Our results support the likelihood that phosphorylation of MDM2 at T216 affects its interactions with proteins. We found that the p53-MDM2 interaction is reduced after
phosphorylation by cyclin A-CDK2. However, the reduction is not as dramatic as reported with DNA-PK phosphorylated N-terminal fragment of MDM2 (53). This is most likely because the DNA-PK phosphorylation site lies within the N-terminal domain of MDM2 that interacts directly with p53, while T216 is outside of the MDM2-p53 interaction domain. Phosphorylation at T216 must therefore have an auxiliary or indirect effect on p53-MDM2 interaction, perhaps through altering the conformation of the MDM2 N-terminus. On the other hand, the interaction between P19ARF-N-37 and MDM2 is enhanced after MDM2 is phosphorylated by cyclin A-CDK2. The effect is quite modest (one third increase in affinity) in our ELISA assay. Other proteins have been reported to interact with the MDM2 acidic domain. Ribosomal L5 protein (81) and the newly reported cell cycle regulating protein MTBP (82) are among candidate proteins we have yet to investigate. Currently, we do not sufficiently understand the properties and functions of MDM2 protein to fully explore the impact of cyclin A-CDK2 phosphorylation.

Taking into account ours and others’ finding, a model emerges for the roles of cyclin A-CDK2 in the relationships between MDM2 and p53. Increased p19ARF-MDM2 interaction, and reduced p53-MDM2 interaction would have a combined effect of inducing the accumulation of free and active p53 protein in the nucleus which in turn would result in increased appearance of p53 effector gene products such as p21. Cyclin A-CDK2 phosphorylation may also increase the activity of p53 protein as evidenced by an earlier observation that phosphorylation of p53 at its CDK site S315 increases its ability to bind to promoter elements of selected target genes, including p21/Waf1 (50). Most speculatively, because phosphorylation of either p53 or MDM2 by cyclin A-CDK2 should have the same result in increasing p53 activity, this may explain why a mutant p53 (S315A) that cannot be phosphorylated by CDKs fails to exhibit significant reduction in transactivation of target promoter reporter constructs (83,84). Increased p53 activity
resulting from cyclin A-CDK2 both activating p53 and releasing p53 from MDM2 would lead to increased synthesis of p21/Waf1. Although p21 works at lower concentrations to inhibit G1 CDKs, it has also been shown to be able, at higher levels, to inhibit cyclin A and cyclin B containing complexes as well (78,85). Thus we propose the existence of a feed-back loop that controls the activity of both cyclin A-CDK2 and p53 when the cells go into S-phase.

Most of our research was done using murine MDM2 and mouse cells. T216 is well conserved in human and mouse MDM2 but neither the CDK consensus sequence nor the CRM is present in human HDM2. Human HDM2 was a much worse substrate than murine MDM2 for cyclin A-CDK2 in our assay (data not shown). We can speculate that the T218 in human HDM2 corresponding to the T216 in murine MDM2 might be recognized by other proline-directed kinases in human cells. There is a cluster of conserved serines or threonines in the vicinity of murine T216 (SESTETPS) and some of these residues may be sites for other serine/threonine kinases. As suggested by our study, phosphorylation at this region has potential impact on the functions of MDM2 protein. Further study on both the regulation and effect of phosphorylation by cyclin A-CDK2 of murine MDM2 should give us more insight into the functions of MDM2 in cellular processes.
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Figure Legends

FIG. 1. Phosphorylation of GST-MDM2 by cyclin A-CDK2.

Full-length GST-MDM2 (100 ng) was used as the substrate in reaction mixtures containing increasing amounts of cyclin A-CDK2 complex. At the highest concentration of the kinase (lane 8), CDK specific inhibitor roscovitine (7 µM) was present in the mixture. GST-MDM2 protein is visualized by Coomassie Blue staining (upper panel), and [\(^{32}\)P] labeled GST-MDM2 is shown in the autoradiogram of the stained gel (lower panel).

FIG. 2. GST-MDM2 is preferentially phosphorylated by cyclin A containing CDK complexes.

Complexes containing cyclin A and CDK2, cyclin E and CDK2, cyclin D1 and CDK4, cyclin B and CDK1, or cyclin A and CDK1 as indicated were used to phosphorylate MDM2 (100 ng), histone H1 (100 ng), or pRb (100 ng) as described in Experimental Procedures. CDK specific inhibitor roscovitine (7 µM) was present in the mixtures at the highest concentration of the kinases containing CDK1 or CDK2. The upper panels (MDM2) show silver stained gels in Part A or Coomassie Blue stained gels in Parts B, C and D. The lower panels are autoradiograms of the indicated [\(^{32}\)P] labeled proteins.

FIG. 3. Mutation at MDM2 T216 abolishes cyclin A-CDK2 specific phosphorylation.

Full-length wild-type or mutant GST-MDM2 as indicated were phosphorylated with cyclin A-CDK2 in vitro and subjected to 2D phospho-peptide mapping using V8 protease. In panel B, the phosphorylation mixtures contained roscovitine (7 µM). The arrow indicates the
major phosphopeptide present in wild-type and T384A MDM2 but absent in either roscovitine containing mixtures or T216A MDM2.

**FIG. 4. MDM2 CRM peptide inhibits cyclin A-CDK2 CRM dependent phosphorylation.**

GST-MDM2 (100 ng), pRb (100 ng) or histone H1 (100 ng) were used as substrates in kinase reaction mixtures containing 0.5 µl cyclin A-CDK2 (see Figure 1). Increasing amounts (to final concentrations of 0.25 µM, 0.5 µM and 1 µM) of the indicated peptides were added to the mixtures. The upper panel shows Coomassie Blue stained GST-MDM2, and the lower panels are the autoradiograms of the [³²P] labeled proteins. CRM sequences identified in the indicated substrate are listed, as well as the sequences for the peptides used in this experiment.

**FIG. 5. MDM2 monoclonal antibody SMP14 cannot recognize GST-MDM2 phosphorylated by cyclin A/CDK2 at T216**

The reactivity of Mab SMP14 with MDM2 protein was determined by ELISA assays (A and B) or Western blot analysis (C, D and E) as described in Experimental Procedures.

A. ELISA assay comparing reactivity of Mab SMP14 in which wells were coated with 200 µl PBS containing the following proteins: BSA (200 µg bovine serum albumin); MDM2 (50 ng GST-MDM2 protein from a kinase reaction mixture containing 200µM ATP but no kinase); MDM2-P (50 ng GST-MDM2 from kinase reaction mixture containing both cyclin A-CDK2 and 200µM ATP); MDM2 (AMP-PNP): (50 ng GST-MDM2 from kinase reaction mixture containing cyclin A-CDK2 and 200µM AMP-PNP).

B. ELISA assay comparing Mab SMP14 reactivity with wild-type and T216A mutant GST-MDM2 proteins. 50 ng or 100 ng of either protein in 200 µl PBS was used to coat each well.
C. GST-MDM2 protein (40 ng) was phosphorylated by increasing amounts of cyclin A-CDK2 (0.5, 1 and 2 µl) and mixtures were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Mab SMP14 or Mab 3G5 as indicated were used to detect GST-MDM2 in the Western blot analysis. Calf intestinal phosphatase (CiP) was added after the kinase reaction to mixtures containing GST-MDM2 at the highest concentration of the kinase.

D. Western blot analysis of Mab SMP14 and Mab 3G5 reactivities with cyclin A-CDK1 (1, 2 and 4 µl) phosphorylated GST-MDM2 as in C.

E. Western blot analysis of Mab SMP14 and Mab 3G5 reactivities with cyclin B-CDK1 (1, 2 and 4 µl) phosphorylated GST-MDM2 as in C.

FIG. 6. Recognition of MDM2 by Mab SMP14 varies with the cell cycle phase.

Swiss 3T3 cells were serum starved for 48 hours and released into serum containing medium. Cells were extracted at different time points after release as indicated. Equal amounts (150 µg) of each extract were run on two gels, which were then transferred to nitrocellulose. Comparably loaded and transferred samples were confirmed by staining the membranes with Ponceau S (not shown). MDM2 antibody Mab SMP14 or Mab 3G5 were used to probe each membrane for MDM2. The cyclin A and cyclin B proteins were detected by H-437 and Ab-2 antibodies respectively. The cell cycle profiles were determined by FACS analysis as summarized in Table 1.

A. Western blots of serum released cell extracts with antibodies as indicated.

B. Ratio of SMP14-reactive MDM2 to 3G5-reactive MDM2 protein at different time points after serum release. The readings for the reactivities were obtained from densitometry analysis on the Western blots shown in part A and blots from a duplicate experiment.
FIG. 7. Effects of cyclin A-CDK2 phosphorylation on the interaction of GST-MDM2 with p53 and p19<sub>ARF</sub> proteins.

Either murine p53 protein (50 ng, A) or p19<sub>ARF</sub>-N-37 peptide (20 ng, C) were coated on each well of a 96-well plate. Different concentrations of wild-type (I) or mutant (T216A, II) GST-MDM2 protein were treated with cyclin A-CDK2 kinase (1.5 µl) in reaction mixtures containing either ATP or AMP-PNP. Mixtures were then diluted in PBS with 10 µM roscovitine, and aliquoted into triplicate wells. The MDM2 monoclonal antibody Mab 4B11 was used to detect the bound MDM2. ∆A<sub>405nm</sub> at each point was calculated from 10-minute interval readings of absorbance at 405nm. Each data point is an average of triplicate experiments.

B. Wild-type GST-MDM2 (400 ng) was treated with cyclin A-CDK2 (1.5 µl) in 30 µl kinase reaction mixtures containing either ATP or AMP-PNP. As a control, GST protein was added in a parallel kinase reaction mixture containing cyclin A-CDK2 and ATP. Either buffer control or murine p53 (50 ng or 100 ng) were incubated with 100 ng of GST-MDM2 from above reaction mixtures bound to Gluthione Sepharose 4B beads (GIBCO-BRL).

TABLE 1. Cell cycle profile of serum-starved and released Swiss 3T3 cells.

Serum starved and released Swiss 3T3 cells were collected in PBS and fixed with cold methanol (-20°C). The cells were then rehydrated in PBS and stained with 60 µg/ml propidium iodide containing 50 µg/ml RNAse A in PBS. The DNA contents of the samples were read on a Becton-Dickinson FACSCalibur system, and the cell cycle profiles were analyzed using ModFit LT software.
**FIG. 1**

|                 | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|-----------------|----|----|----|----|----|----|----|----|
| **Cyclin A-CDK2(μl)** | 4  | -  | 0.25 | 0.5 | 1  | 2  | 4  | 4  |
| **Roscovitine**  | -  | -  | -  | -  | -  | -  | -  | +  |
| **MDM2**         |    |    |    |    |    |    |    |    |
| **[^32P]MDM2**   |    |    |    |    |    |    |    |    |
### FIG. 2

**A**

|       | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|-------|---|---|---|---|---|---|---|---|---|
| Cyclin A-CDK2($\mu$l) | - | 0.25 | 0.5 | 1 | 1 | - | - | - | - |
| Cyclin E-CDK2($\mu$l) | - | - | - | - | - | 0.3 | 0.6 | 1.2 | 1.2 |
| Roscovitine | - | - | - | - | + | - | - | - | + |
| MDM2 | ![MDM2](image1) |
| $[^{32}P]$ MDM2 | ![MDM2](image2) |
| $[^{32}P]$ histone H1 | ![Histone H1](image3) |

**B**

|       | 1 | 2 | 3 | 4 |
|-------|---|---|---|---|
| Cyclin A-CDK2($\mu$l) | 0.13 | 0.26 | - | - |
| Cyclin D1-CDK4($\mu$l) | - | - | 0.5 | 1 |
| MDM2 | ![MDM2](image4) |
| $[^{32}P]$ MDM2 | ![MDM2](image5) |
| $[^{32}P]$ pRb | ![pRb](image6) |
### C

|                      | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|----------------------|---|---|---|---|---|---|---|---|
| **Cyclin A-CDK2(µl)**|   |   |   | 0.25 | 0.5 | 1 | 1 | - |
| **Cyclin B-CDK1(µl)**|   |   |   |   |   |   |   | 0.25 | 0.5 | 1 | 1 |
| Roscovitine          |   |   |   |   |   |   |   | + |
| MDM2                 |   |   |   |   |   |   |   |   |
| [³²P] MDM2           |   |   |   |   |   |   |   |   |
| [³²P] histone H1      |   |   |   |   |   |   |   |   |

### D

|                      | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|----------------------|---|---|---|---|---|---|---|---|---|
| **Cyclin A-CDK1(µl)**|   |   |   | 0.5 | 1 | 2 | 2 | - | - |
| **Cyclin B-CDK1(µl)**|   |   |   |   |   |   |   | 0.25 | 0.5 | 1 | 1 |
| Roscovitine          |   |   |   |   |   |   |   | + |
| MDM2                 |   |   |   |   |   |   |   |   |
| [³²P] MDM2           |   |   |   |   |   |   |   |   |
| [³²P] histone H1      |   |   |   |   |   |   |   |   |
FIG. 3

A
Wild-type

B
Wild-type + Roscovitine

C
T216A

D
T384A
FIG. 4

|    | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|----|---|---|---|---|---|---|---|---|---|-----|----|
| Cyclin A-CDK2 | - | + | + | + | + | + | + | + | + | +   | +  |
| Peptide (μM)  | - | - |   |   |   |   |   |   |   |     |     |
| CRM          | 0.25 | 0.5 | 1  | 0.25 | 0.5 | 1  | 0.25 | 0.5 | 1   |     |
| CRM-S        |     |     |     |     |     |     |     |     |     |     |     |
| CDK          |     |     |     |     |     |     |     |     |     |     |     |

MDM2

[32P]MDM2

[32P]pRb

[32P]histone H1

**CRM consensus:**

|    | ZRXL |
|----|------|
| p107 | SAKRRRLFG |
| p21  | HSKRRRLIF |
| p57  | SACRSLFG |
| E2F1 | PVKRRDL |
| pRb  | KPLKLRFL |
| p53  | SRHKLMLF |
| PAP  | SKIRILVG |

**MDM2 peptides**

|    |     |
|----|-----|
| CRM: | RKRRRLS_SFDPCA |
| CRM-S: | LRPKSFRRDSRRA |
| CDK: | SESTETPSHQDL |
FIG. 6

| Hours after serum release | 0  | 8  | 14 | 16 | 18 | 20 | 24 |
|---------------------------|----|----|----|----|----|----|----|
| MDM2                      |    |    |    |    |    |    |    |
| SMP14                     |    |    |    |    |    |    |    |
| 3G5                       |    |    |    |    |    |    |    |
| Cyclin A                  |    |    |    |    |    |    |    |
| Cyclin B                  |    |    |    |    |    |    |    |
| Cell Cycle Phase          | G0 | G1 | S  | G2/M |
Fig 7

A

I

II

ΔA_{405nm} vs MDM2 (ng)

- P53+MDM2-P
- P53+MDM2 (AMPPNP)

ΔA_{405nm} vs MDM2 (ng)

- P53+ T216A-P
- P53+T216A (AMPPNP)
FIG. 7

B

|     | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|-----|-----|-----|-----|-----|-----|-----|-----|
| **Bound on GST-beads:** | GST | GST-MDM2 | GST-MDM2-P |
| **p53 (ng)** | 100 | 0 | 50 | 100 | 0 | 50 | 100 |

![Image of gel showing protein bands for MDM2 and p53]
| Time after Serum Release | Cell Cycle Profile |   |
|--------------------------|--------------------|---|
|                          | G0/G1              | S |
| 0 hours                  | 84%                | 3%|
| 8 hours                  | 84%                | 3%|
| 14 hours                 | 72%                | 20%|
| 16 hours                 | 52%                | 39%|
| 18 hours                 | 27%                | 59%|
| 20 hours                 | 9%                 | 70%|
| 24 hours                 | 15%                | 36%|
Cyclin A-CDK phosphorylation regulates MDM2 protein interactions
Tingting Zhang and Carol Prives

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