Posttranslational Modification of MDM2

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Abstract

The functions of the MDM2 protein, in particular its E3 ubiquitin ligase activity and its ability to interact with a number of cellular proteins intimately involved in growth regulation, are modulated by sumoylation and multisite phosphorylation. These posttranslational mechanisms not only regulate the intrinsic activity of MDM2 in response to cellular stresses, but also govern its subcellular localization, differentiate between MDM2-mediated ubiquitination of p53 and autoubiquitination, integrate the stress response with mechanisms that mediate cell survival, and modulate the interaction of MDM2 with cellular and viral proteins. In this review, we summarize our current knowledge of the role of posttranslational modifications of MDM2 and their functional relevance.

Introduction

MDM2

The MDM21 protein is overexpressed in a significant number of human tumors underscoring its pivotal involvement in the development of human disease (1–6). The principal function of MDM2 is that of an E3 ubiquitin ligase which, together with the p300 “transcriptional co-activator” protein (in its capacity as an E4 ligase), mediates the ubiquitination and proteasome-dependent degradation of the p53 tumor suppressor protein and other growth regulatory proteins (7–9). In addition to mediating degradation of p53, MDM2 blocks the interaction of p53 with the transcriptional apparatus (10), mediates translocation of p53 to the cytoplasm (11), thereby removing it from its site of action, and recruits the histone deacetylase HDAC1 to deacetylate key lysine residues in the COOH terminus of p53 thus making them available for ubiquitination (12). Overproduction of MDM2 is therefore thought to suppress normal p53 levels and stifle the p53 response to cellular stress.

In addition to p53, many other MDM2-interacting proteins (including the Rb and ARF tumor suppressors) have been identified. Moreover, functions of MDM2 which are independent of p53 and may contribute to its high oncogenic potential, have been established (13).

MDM2 is a nuclear phosphoprotein that contains several conserved functional regions. The NH2 terminus contains the p53-interacting domain, located between residues 23–108 (14, 15). Within the central domain, there are binding sites for TBP (16), p300 (8, 9), and ARF (17), one of two products encoded by overlapping reading frames within the INK4A gene, which binds to MDM2 and blocks it ability to mediate ubiquitination of p53 (but does not affect autoubiquitination; hereafter ARF). The central region also contains nuclear localization and export sequences which mediate nuclear-cytoplasmic shuttling and a zinc finger domain which overlaps with the binding site for the Rb tumor suppressor protein. The COOH terminus of MDM2 contains a RING finger domain which is required for MDM2-mediated ubiquitin transfer (18–20; Fig. 1).

Ubiquitination of MDM2

MDM2 possesses the activity of an E3 ubiquitin ligase which mediates autoubiquitination as well as the ubiquitination of other substrates including p53 (18, 20, 21). Interestingly, MDM2 mediates multiple monoubiquitin attachment to p53 (22) while p300 mediates subsequent polyubiquitination (9). The E3 activity of MDM2 is dependent on its RING finger domain and is abolished by mutations which delete the domain or substitute any of the amino acids required for the coordination of zinc (18, 21). Consistent with loss of ubiquitination function, these mutations stabilize MDM2 and p53 in cells. Specificity in the transfer of ubiquitin to p53 and MDM2 itself resides, in part, in the RING domain and substitution of this domain with a heterologous RING finger permits autoubiquitination but abolishes ubiquitination of p53 (18). While sites of ubiquitination have been mapped to a cluster of lysine residues in the COOH terminus of p53 (23), the identities of the autoubiquitination sites in MDM2 have not been published.

The balance between auto- and substrate-ubiquitination of MDM2 is modulated physiologically by posttranslational modifications, including sumoylation and phosphorylation. On SUMO conjugation to MDM2, its E3 ligase activity is shifted toward p53, while self-ubiquitination is minimized (24). DNA damage and other stresses reduce the ability of MDM2 to interact with p53 which leads to stabilization and activation of the transactivation function of p53 (25). Although it has not been demonstrated directly in all cases, the outcome of uncoupling...
p53 from MDM2 will be reduced p53 ubiquitination. Mechanisms by which stresses uncouple these two proteins include abrogation of MDM2 expression and phosphorylation of p53 at the NH2 terminal sites S15, T18, and S20 (reviewed in 7, 26). Phosphorylation of MDM2 itself at S395 can attenuate p53 degradation and is likely to integrate with these other events. Moreover, other phosphorylation events within the MDM2 molecule may independently regulate the E3 activity of MDM2. It has also been suggested that acetylation of lysine residues may down-regulate the E3 activity of MDM2 (7). Finally, protein-protein interactions, such as the MDM2-ARF interaction, also have profound effects on MDM2 E3 ligase activity. These effects have been reviewed extensively by others (e.g., see 7, 27).

**Sumoylation of MDM2**

Reversible modification of a number of proteins involved in gene expression by the small ubiquitin-like modifier, SUMO, can have profound effects on stability, localization, protein-protein interaction, DNA binding, and activation. Accordingly, sumoylation is emerging as an important component of the regulatory apparatus that integrates the control of gene expression (28). Mammals have three types of SUMO [SUMO-1, -2, and -3] and the sumoylation pathway is strikingly similar to the ubiquitination pathway. MDM2 is sumoylated (SUMO-1) in vivo by the SUMO E3 ligases Ubc9, PIAS1, and PIASxβ, and can be also sumoylated in vitro by these enzymes or by RanBP2 (24, 29). The interaction with Ubc9 requires amino acids 40–59 in MDM2 both in vitro and in vivo and Ubc9-dependent sumoylation of MDM2 can be blocked using a peptide corresponding to this sequence (24). Identification of the sumoylation site(s) has been difficult because of the fact that one of the potential lysine targets, lys182, is located within the nuclear localization sequence and mutation of this residue confines MDM2 to the cytoplasm where it is spatially separated from the modifying enzymes (29). Current evidence suggests that the site(s) of SUMO modification lies within amino acids 134–212, a region which contains the lys182 residue as well as lysines 136, 146, and 185 (30). One model proposes that MDM2 becomes sumoylated as it enters the nucleus, because RanBP2 is part of the nuclear pore, and further sumoylated by PIAS proteins within the nucleus itself (29). The factors/signals that lead to sumoylation of MDM2 are still unclear; however, one striking observation is that MDM2 sumoylation is stimulated significantly by ARF (30). This is particularly interesting given that ARF blocks the ubiquitination of p53 by MDM2 and is consistent with the idea that SUMO and ubiquitin modifications may be mutually exclusive and/or antagonistic. Also in keeping with this idea, expression of a mutant Ubc9 protein leads to down-regulation of MDM2 sumoylation in a dominant negative manner, coupled with a corresponding increase in MDM2 ubiquitination and decrease in p53 ubiquitination (24). These data suggest that SUMO-1 modification of MDM2 can differentially modulate

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**FIGURE 1.** Schematic diagram showing the functional domains and phosphorylation events impinging on MDM2. The amino acid residues are numbered. Functional domains of the MDM2 protein including the p53 binding domain, nuclear localization sequence (NLS), nuclear export sequence (NES), acidic domain, zinc finger, RING finger, and nucleolar localization sequence (NoLS) are indicated. The positions of the phosphorylation-sensitive epitopes for monoclonal antibodies SMP14 and 2A10 are also given. Phosphorylation sites are indicated by the letter P within an ellipse. Their locations relative to the functional domains of MDM2 are shown. Protein kinases, where known, are indicated in boxes with the target residue(s) shown above. The effects of individual phosphorylation events are indicated: a vertical arrow signifies stimulation, whereas an inverted “T” indicates inhibition.
the outcome of MDM2 E3 ligase activity in a manner that favors p53 accumulation. This switch in modification status is stress-responsive, because UV irradiation leads to a decrease in the interaction of MDM2 with Ubc9 and a corresponding loss of MDM2 sumoylation (24).

**Multisite Phosphorylation of MDM2**

The first demonstration of the complex and multisite nature of MDM2 phosphorylation was by Henning et al. (31) who showed that the phosphorylation status of MDM2 is influenced by early gene expression of SV40, with the MDM2 from the SV40-infected or transformed cells showing both the appearance of a novel group of phosphopeptides and the disappearance of one major phosphopeptide present in MDM2 from the normal cells. This study also indicated that, like p53, MDM2 is stabilized in the presence of SV40. Moreover, hyperphosphorylated MDM2 participates in a triple complex with p53 and T antigen (T-Ag, the transforming protein of SV40), which is thought to activate oncogenic functions of MDM2 and enhance the transforming potential of T-Ag (31). These data therefore suggest the possibility that SV40-dependent differences in the phosphorylation status of MDM2 may be important for cellular transformation.

Almost 20% of the amino acids on the MDM2 protein are either serine or threonine residues, and the MDM2 protein is phosphorylated at multiple sites in vivo. Two clusters of phosphorylation sites are located at the NH2 terminal (amino acids 1–193) and central (amino acids 194–293) domains of murine Mdm2, respectively (32). Mapping of these clusters fits well with more recent studies (carried out for the most part with human MDM2) which have identified a number of sites that are phosphorylated in a cellular context, including (with the modifying enzymes, where known, given in parentheses) ser166 (Akt), ser186 (Akt), thr219 (cyclin A-CDK1/2), ser229, ser232, ser240, ser242, ser246, ser253, ser256, ser260, ser262, ser269 (CK2), tyr294 (c-Abl), and ser295 (ATM [‘‘ataxia telangiectasia-mutated’’ protein kinase]). In addition to these well-characterized two other phosphorylation sites in this region, threonine 168 (human MDM2; 33) and serine 189 (murine Mdm2; 32), have been suggested on the basis of salt bridge formation with residues proximity to p53 residues thr18 and ser20, both of which weaken the MDM2-p53 association in their phosphorylated states. Simultaneous phosphorylation of all of these residues is predicted to have a significant influence in disrupting MDM2-p53 contact, while phosphorylation of ser17 regulates MDM2-p53 association in a cellular context, but this has yet to be demonstrated. Similarly, it will be of considerable interest to learn whether this modification, and its biological effects, occur in response to DNA damage, and whether ‘‘natural’’ promoters (such as p21, Bax PIG3, etc.) are stimulated as a consequence of the phosphorylation, leading to growth arrest or apoptosis.

Recent analysis of the structure of the NH2 terminus of MDM2, as determined by nuclear magnetic resonance, has added weight to the potential for ser17 as a regulatory site of modification (37). Amino acids 25–109 of MDM2 form an ordered structure containing a hydrophobic cleft which accommodates p53 amino acids 19–26. McCoy et al. (37) suggest that amino acids 16–24 of MDM2 can form a ‘‘flexible lid’’ that folds over and stabilizes the MDM2 structure but competes only weakly with p53 for binding to this cleft. Strikingly, the predicted structure places MDM2-ser17 in close proximity to p53 residues thr18 and ser20, both of which weaken the MDM2-p53 association in their phosphorylated states. Simultaneous phosphorylation of all of these residues is predicted to have a significant influence in disrupting MDM2-p53 contact, while phosphorylation of MDM2-ser17 itself is additionally proposed to stabilize interaction of the lid with MDM2 on the basis of salt bridge formation with residues his73 and lys94. In support of these ideas, the lid of a S17D mutant was found to have higher affinity for MDM2 than the wild-type protein. However, it has yet to be determined whether phosphorylation per se will directly mediate these effects.

**Phosphorylation of the p53 Binding Domain in MDM2 by DNA-PK**

The DNA-activated protein kinase (DNA-PK) is a member of the phosphatidylinositol 3-kinase (PI3-K) family which includes the ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related) protein kinases. DNA-PK, ATM, and ATR share many substrates (at least in vitro) and are each targeted toward an SQ core motif. MDM2 is phosphorylated in vitro by both DNA-PK (34) and ATM (35) but phosphorylation by ATR has not yet been reported. Of eight potential DNA-PK targets in MDM2, only ser17 has, to date, been shown to be phosphorylated by this enzyme in vitro (34). Like DNA-PK, ATM is believed to phosphorylate several sites in MDM2 in vitro, including an, as yet, unidentified site within the amino terminal 115 residues (which, incidentally, contain only one SQ motif, located at position 17; 35, 36). Interestingly, however, although physiological phosphorylation of ser-17 has yet to be confirmed, the phosphorylation site itself has been reported to have a significant impact on the ability of MDM2 to regulate the p53 response. For example, ELISA analysis has shown that phosphorylation of MDM2-ser17 can block the MDM2-p53 interaction in vitro, whereas the ability of a S17A mutant of MDM2 to associate with p53 is unaltered following phosphorylation by DNA-PK (34). Consistent with this observation, an S17A mutant was significantly more effective in inhibiting p53-dependent transactivation (using the PG13-CAT report plasmid) in cultured cells than wild-type MDM2, presumably because its association with p53 cannot be weakened by ser17 phosphorylation. These data are in keeping with the notion that phosphorylation of ser17 regulates MDM2-p53 association in a cellular context, but this has yet to be demonstrated. Similarly, it will be of considerable interest to learn whether this modification, and its biological effects, occur in response to DNA damage, and whether ‘‘natural’’ promoters (such as p21, Bax PIG3, etc.) are stimulated as a consequence of the phosphorylation, leading to growth arrest or apoptosis.

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**Regulation of MDM2 by ATM**

Recent studies have led to the conclusion that targeting of p53 by ATM may be only part of the p53 induction mechanism and that rapid ATM-dependent phosphorylation of MDM2 is likely to play a critical role in this process (35, 36, 38). ATM is able to phosphorylate MDM2 at ser395 in vitro, within one of two core motifs (comprising the sequence DYS) for the mononuclear antibody, 2A10. Consistent with the identification of this site, a decrease in 2A10 reactivity was observed to occur rapidly, and in an ATM-dependent manner, following exposure of cells to genotoxic stress but this was not seen with a S395A

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mutant. Similarly, the importance of this site was underscored by the observation that microinjection of 2A10, but not a control antibody, is sufficient to induce p53. From a functional standpoint, a S395D mutant (where the substitution of asp for ser was carried out to mimic the negative charge provided by a phosphate) could only degrade p53 weakly in comparison to wild-type MDM2 or a S395A mutant, and was unable to promote nuclear export of p53. These data are consistent with a model in which direct phosphorylation of MDM2 by ATM inhibits its ability to mediate p53 turnover. The mechanism of this effect does not appear to involve changes in association with p53, but may involve phosphorylation-dependent regulation of interaction with the nuclear export machinery. It is also possible that other factors such as ubiquitination of p53 in vivo and/or association with p300 play a role in this mechanism but this remains to be clarified.

An interesting twist to the loss of 2A10 reactivity is that the ATM pathway also promotes the loss of the epitope for a second monoclonal antibody, SMP14 (38). The loss of this epitope involves a phosphorylation event but it is unlikely that this is mediated directly by ATM. SMP14 interacts with amino acids 154–167 in human MDM2 and includes ser166, one of the two Akt-targeted sites. SMP14 reactivity is also influenced both by phosphorylation of thr216 and amino acid substitution of thr216, suggesting that its epitope may be more extensive than simply amino acids 154–167. It is therefore possible that ATM may not only phosphorylate MDM2 directly, but could indirectly regulate the phosphorylation of other residues in MDM2. The p53 protein, which is phosphorylated and activated by the ATM protein kinase in vivo, a S395D mutant was shown to increase MDM2-mediated p53 degradation, stimulate MDM2-mediated down-regulation of p53-dependent transactivation, and moderately enhance the ability of MDM2 to inhibit p53-mediated apoptosis. The data are in keeping with a model in which DNA damage-dependent phosphorylation of MDM2 by c-Abl contributes to apoptosis by blocking the ability of MDM2 to down-regulate p53 function. The link between MDM2 and c-Abl is also striking in that the phosphorylation of MDM2 is mediated by a protein kinase which is in itself regulated through phosphorylation by ATM. Therefore, like p53, ATM cannot only directly phosphorylate MDM2 itself, but additionally can indirectly regulate the protein by activating another MDM2 kinase. Also striking is the finding that the target of c-Abl, tyr294, is immediately adjacent to the ATM target, ser395. These observations raise the interesting possibility that ATM may require “two hits” to inhibit MDM2, perhaps as a safeguard when activating a potent antiproliferative or antisurvival response, or as a means of amplifying the response.

**Regulation of MDM2 by the c-Abl Protein Tyrosine Kinase**

The c-Abl proto-oncogene encodes a protein-tyrosine kinase that can shuttle between cytoplasmic and nuclear compartments. Cytoplasmic c-Abl is intimately involved in mediating growth and survival signals (including those mediated by the PI3-K, STAT5, and Ras/ERK pathways). In contrast, however, nuclear c-Abl plays a pivotal role in mediating apoptosis (39) and is phosphorylated and activated by the ATM protein kinase in response to genotoxic agents (40, 41). Part of the mechanism(s) by which nuclear c-Abl can induce apoptosis involves phosphorylation and activation of the p53-related protein, p73 (42). However, c-Abl interacts with other nuclear proteins and a direct link between c-Abl and MDM2 was suggested by the observations that c-Abl can block ubiquitination and nuclear export of p53, coupled with the finding that c-Abl-null cells fail to accumulate p53 efficiently after DNA damage (43, 44).

Building on these preliminary studies, Goldberg *et al.* (45) have now shown that MDM2 and c-Abl associate both *in vitro* and in the nuclei of cultured cells leading to the phosphorylation of MDM2 at multiple sites. Tyr394 was proposed and subsequently identified as a key site of c-Abl-dependent phosphorylation in this study, based on its similarity to a c-Abl consensus target sequence and to the c-Abl phosphorylation site in p73. A phospho-specific antibody directed against Y394, coupled with the use of a Y394F mutant that cannot be phosphorylated, confirmed that this site is phosphorylated physiologically in a c-Abl-dependent manner. The Y394F mutant was shown to increase MDM2-mediated p53 degradation, stimulate MDM2-mediated down-regulation of p53-dependent transactivation, and moderately enhance the ability of MDM2 to inhibit p53-mediated apoptosis. The data are in keeping with a model in which DNA damage-dependent phosphorylation of MDM2 by c-Abl contributes to apoptosis by blocking the ability of MDM2 to down-regulate p53 function. The link between MDM2 and c-Abl is also striking in that the phosphorylation of MDM2 is mediated by a protein kinase which is in itself regulated through phosphorylation by ATM. Therefore, like p53, ATM cannot only directly phosphorylate MDM2 itself, but additionally can indirectly regulate the protein by activating another MDM2 kinase. Also striking is the finding that the target of c-Abl, tyr294, is immediately adjacent to the ATM target, ser395. These observations raise the interesting possibility that ATM may require “two hits” to inhibit MDM2, perhaps as a safeguard when activating a potent antiproliferative or antisurvival response, or as a means of amplifying the response.

**Regulation of MDM2 by Phosphorylation Mediated by the PI3-K/Akt Pathway**

Many growth factors and cytokines not only promote cell proliferation but can also maintain cell viability. The binding of these factors to their receptors initiates a signaling cascade leading to the activation of the lipid kinase, PI3-K, and the generation of the second messenger phosphatidylinositol (3,4,5)-(trisphosphate (PIP3; reviewed by Refs. 46, 47). PIP3 recruits protein kinases containing pleckstrin homology domains to the membrane, including Akt (also known as protein kinase B) and its upstream activators PDK1 and PDK2. Phosphorylation of Akt at serines 308 and 473 activates the kinase and permits its release from the membrane whereupon it can interact with, and phosphorylate a range of cytoplasmic and nuclear substrates including IKK, p21WAF1/CIP1, p27, and forkhead. Coordinate regulation of these different proteins thus leads to an integrated response that disfavors apoptosis and promotes cell survival. PDK1 and PDK2 are also activators of other protein kinases including p90RSK which integrates signals transduced through the PI3-K and ERK pathways, and p70S6K.

A model has emerged recently describing the mechanism by which survival signals influence the p53 response through regulation of MDM2 (outlined in Fig. 2; 33, 48–52). According to this model, MDM2 associates with Akt and, following activation of the kinase, or ectopic expression of constitutively active Akt, serine residues 166 and 186 in MDM2, both of which lie within RXRXXS/T consensus motifs (for Akt and several other AGC kinases), become phosphorylated (33, 48–52). These amino acids lie within close proximity of two nuclear localization sequences (NLS) and a nuclear export sequence (NES) in MDM2, and the evidence suggests that phosphorylation of these serines 166 and 186 by Akt stimulates entry into the nucleus (33, 52). In
The influence of this pathway on the mechanisms governing the activity of p53 is the integrated response achieved through the disruption of PI3-K/Akt signaling. This process can be blocked by the agents that dephosphorylate PIP3, or treatment of cells with the PI3-K inhibitor LY294002, each leads to dephosphorylation of MDM2 at ser166 and ser186 and inhibition of entry of MDM2 into the nucleus (33, 50, 52). Substitution of these phosphoacceptor residues may block nuclear export. Similarly, phosphorylation of this site could regulate attachment of MDM2 to a cytoplasmic anchor, a scenario that is plausible given the recent observation that the Akt pathway governs this function but its influence is 2-fold. Firstly, phosphorylation of the receptor itself (at serines 248 and 246) increases its binding to MDM2. Phosphorylation also permits nuclear localization of MDM2 following disruption of Akt signaling (33, 52), other groups have reported that MDM2 is localized within the nucleus irrespective of the presence or absence of Akt-dependent phosphorylation (48, 51). Again, the reasons for these disparate observations are unclear, especially given that similar cell lines and procedures were carried out in the different studies.

There also remains the unanswered question concerning the mechanism of the regulation. If Akt-dependent signaling does indeed govern MDM2 subcellular localization, how is this brought about? Given that most well-characterized examples of phosphorylation on the COOH terminal side of classical NLSs actually block, rather than promote, nuclear entry (e.g., p21, forkhead, SV40 T-Ag), it seems unlikely that subcellular localization of MDM2 is governed by the same mechanism. One possible explanation is that because the ser186 site is also located on the immediate NH2 terminal flank of the NES of MDM2, the phosphorylation of this residue may block nuclear export. Similarly, phosphorylation could regulate attachment of MDM2 to a cytoplasmic anchor, a scenario that is plausible given the recent observation that p53 is retained in the cytoplasm through association with PARC (57). Future studies will no doubt resolve this issue.

Finally, the regulatory influence of MDM2 and Akt is not solely confined to the p53 pathway, as recent evidence indicates that MDM2 can also mediate ubiquitination and degradation of the androgen receptor (AR; 58). As with p53, the Akt pathway governs this function but its influence is 2-fold. Firstly, phosphorylation of the receptor itself (at serines 210 and 690) increases its binding to MDM2. Phosphorylation of MDM2 at ser166 and ser186 is then required for the subsequent ubiquitination and degradation of the AR protein.

FIGURE 2. Responsiveness of MDM2 to survival signaling. Survival factors acting through the PI3-K pathway lead to the phosphorylation and activation of the protein kinase Akt/PKB. According to the current model, Akt associates with MDM2 in the cytoplasm. Once activated, Akt phosphorylates MDM2 and promotes its entry into the nucleus. The phosphorylated MDM2 associates with p300, stimulates the degradation of p33, and blocks the inhibition of MDM2 by ARF. Details of this mechanism are given in the text.
The biological consequence of these regulatory events is that MDM2 can down-regulate expression from AR-dependent promoters and inhibit suppression of colony formation in a manner that is dependent on Akt and the presence of the ser166/186 phosphorylation sites in MDM2. The data support the idea that MDM2 is likely to coordinate the control of several proteins with key roles in governing cell growth and apoptosis.

Cell Cycle Regulation of MDM2

Evidence has emerged that cell cycle transition itself may play an important role in regulating MDM2 activity. Zhang and Prives (59) have shown that cyclin A-CDK2 phosphorlates murine Mdm2 in vitro at a single residue, threonine 216, and that a cyclin-CDK substrate recognition motif (CRM) is present in the sequence RRSLL (amino acids 181–184 of murine Mdm2). A peptide representing CRM of MDM2, but not a scrambled peptide nor a peptide corresponding to the sequence containing the phosphorylation site, is able to inhibit MDM2 phosphorylation by cyclin A-CDK2, underscoring the importance of the motif. The specificity of this reaction is also striking because phosphorylation of MDM2 can be achieved by cyclin A-CDK2 and, to a lesser extent, cyclin A-CDK1, but not by any of the other cyclin-CDK complexes. It is significant, however, that the CRM is not present in human MDM2, nor is the human protein a good substrate for cyclin A-CDK2. Given the potent phosphorylation of murine Mdm2 by this enzyme, it is entirely possible that thr216 phosphorylation may be specific to the CRM is present in the sequence (ser183) is phosphorylated by Akt in response to survival stimuli. The question then arises as to whether Akt phosphorylation of MDM2 may influence its subsequent phosphorylation by cyclin A-CDK2, but the answer to this question is not known at present.

Zhang and Prives (59) also find that loss of reactivity with the monoclonal antibody SMP14 is a consequence of cyclin A-CDK2-mediated phosphorylation of MDM2, even though the phosphorylation site and the SMP14 epitope are separated by about 50 amino acids. It is highly unlikely that there is a second phosphorylation site within the SMP14 epitope because this does not contain a consensus sequence for CDK phosphorylation. However, the finding that SMP14 shows reduced reactivity with a T216A mutant of MDM2 would be consistent with the idea that there are additional elements to the epitope that may include thr216. On the basis of reactivity of this antibody with MDM2, thr216 becomes phosphorylated at G1/S boundary, an event which coincides with the synthesis of cyclin A and a corresponding phosphorylation-dependent shift in the migration of MDM2 observed during gel electrophoresis. It should be remembered, however, that loss of the SMP14 epitope can also occur through the ATM pathway in response to DNA damage (38) and therefore the loss of SMP14 reactivity could involve additional events.

Functionally, thr216 phosphorylation appears to weaken the MDM2-p53 interaction and strengthen the association of MDM2 with ARF. These effects are subtle but perhaps this is appropriate because one would not wish to elicit a strong p53 response that would prevent the cells from traversing S phase. What then would be the purpose or outcome of providing a modest induction of p53? One proposed idea is that elevated p53 may increase the levels of p21WAF1 leading to a feedback control on cyclin A-CDK2 activity as cells enter S phase (59). Another possibility is that thr216 phosphorylation may synergize with other modifications in MDM2. For example, as with thr216 phosphorylation, in vitro phosphorylation of s17 contributes to the disruption of MDM2-p53 association. Phosphorylation of a site such as thr216, which can independently regulate p53 contact, may synergize with ser17 phosphorylation to bring about a more robust p53 induction under a given set of circumstances. Another possibility is that phosphorylation of MDM2 by cyclin A-CDK2 may influence the established ability of MDM2 to regulate cyclin A expression. Interesting as these ideas may be, they will have to be tested directly.

Multisite Phosphorylation of the Acidic Domain

The acidic domain of MDM2 comprises amino acids 235–295 and contains structural elements that contribute to the interaction with p300, ARF, and Rb. Deletion mutants that remove amino acid sequences flanking, or from within, the acidic region are still capable of autoubiquitination and ubiquitination of p53, but strikingly, fail to degrade MDM2 and p53 (60, 61). Loss of the ability to degrade p53 correlates with loss of p300 binding (61), consistent with the idea that p300-mediated polyubiquitination of monoubiquitinated p53 is required for degradation of p53 by the proteasome (9). The acidic domain is therefore an integral and important functional element in at least one, and probably in several, biochemical functions of MDM2.

A cluster of phosphorylation sites observed in the acidic domain has recently been shown to contain phosphorylated serines 240, 242, 246, 253, 256, 260, and 262 (32, 62). The data suggest that this cluster of residues is normally phosphorylated in the cell under nonstressed conditions (62), but the identity of the protein kinase(s) responsible for modifying these sites in vivo is entirely unclear at present. Functionally, however, modification of each of these residues may contribute significantly to promoting MDM2-mediated p53 turnover. This conclusion is based on the use of a series of mutants (in both human and murine Mdm2) in which serine to alanine substitutions, either singly or in pairs, alleviated from various degrees, degradation of p53 in transfection experiments (62). Strikingly, as with the deletion mutants of the acidic domain, the ability of each of the mutant proteins to ubiquitinate p53 (and autoubiquitinate) was unaffected, indicating that the serine residues play an important role in governing a postubiquitination function of MDM2 that is essential for degradation. The most likely function is the interaction of a partner protein, possibly p300, but this remains to be determined. Accordingly, phosphorylation of the serine residues would be expected to contribute, each in turn and to varying degrees, to association with the partner. Dephosphorylation of these residues in response to an appropriate signal would lead to weakened association with the partner and accumulation of p53 (Fig. 3).
Phosphorylation of MDM2 by Protein Kinase CK2

Guerra et al. (63) showed that MDM2 can be phosphorylated by both the holoenzyme and catalytic subunit of protein kinase CK2 in vitro. Interestingly, stimulation of holoenzyme-dependent MDM2 phosphorylation by polylysine could be mimicked by full-length p53 or by the COOH terminus of p53 (amino acids 264–393), suggesting a potential physiological role for p53 in mediating MDM2 phosphorylation. Subsequent studies have independently identified ser267 of murine Mdm2 (equivalent to ser269 in human MDM2) as the major site of phosphorylation by CK2 (64, 65) while ser258 has been identified as a minor CK2 target (65). Phosphorylation of ser267 by CK2 occurs physiologically and, apart from a minor role in stimulating turnover of p53 by MDM2 (65), the physiological relevance of this phosphorylation event is presently unclear. Recently, it has been shown that a protein kinase complex comprising CK2 and the transcriptional elongation factor FACT is activated on UV irradiation, leading to the phosphorylation of p53 at Ser392 (66), an event which stimulates the site-specific DNA-binding function of p53 (67). Surprisingly, this study indicated that while the CK2/FACt complex, as compared with recombinant CK2 holoenzyme, preferentially targeted p53, it was relatively less active toward MDM2 and other established CK2 substrates (66). On the basis of this study, it would seem that coordinate regulation of both p53 and MDM2 by CK2 is perhaps unlikely. Determination of the responsiveness of ser267 phosphorylation to UV, and other stresses, and investigation of the functional consequence of ser267 phosphorylation will, perhaps, shed light on the role of CK2 in regulating MDM2.

Regulation of MDM2 by Stress-Induced Dephosphorylation

Protein dephosphorylation contributes significantly to the mechanism of p53 induction in response to cellular stress. For example, p53-serine 376 dephosphorylation was reported to occur in response to DNA damage, and in an ATM-dependent fashion, leading to the interaction of 14-3-3 with a cryptic binding site on p53, and stimulation of p53 site-specific DNA binding (68). Growing evidence suggests that dephosphorylation of MDM2 is also likely to be a critical event following stress and there are now two striking examples of mechanisms where MDM2 dephosphorylation plays a key role in the p53 response.

FIGURE 3. Stress-responsive dephosphorylation of the acidic domain of MDM2. MDM2 promotes the ubiquitination and degradation of p53 in a manner that requires the p300 co-activator protein. p53 degradation is stimulated by a cluster of phosphorylation events within the central acidic domain of MDM2. Dephosphorylation of these residues occurs in response to ionizing radiation and blocks degradation, but not ubiquitination of p53. It is not known at present whether this involves inactivation of a protein kinase(s), activation of a protein phosphatase(s), or both of these events. Further details of this model are given in the text.
The first of these involves the cyclin G1 protein, the product of one of the first p53 responsive genes to be identified (69); (cyclin G1 has two homologues, cyclins G2 and I). The significance of cyclin G1 induction in the p53 response was unclear initially because it appeared to be involved in growth promotion functions and was found to be highly expressed in regenerating tissues and tumors, roles that might appear to be inconsistent with an involvement in mediating the p53 response (70). However, insight into the function of cyclin G1 came from the identification of the B' subunit of protein phosphatase 2A subunit (PP2A) as an interacting partner for cyclin G1 in the yeast two-hybrid system (71). PP2A is involved in a broad spectrum of cellular events but its specificity is mediated through a series of regulatory subunits of which there are three classes, termed B, B', and B". These interact with common catalytic (C) and scaffolding (A) subunits. Two recent studies (72, 73) have demonstrated that cyclin G1 interacts directly with MDM2 and with PP2A holoenzyme encompassing the B' subunit. In this capacity, cyclin G1 can act both as a targeting subunit and a selectivity factor that stimulates PP2A activity toward MDM2. Cyclin G1 is also thought to play an additional role in regulating MDM2 by promoting MDM2-ARF association (72).

The finding that cyclin G1-PP2A dephosphorylates MDM2 residue thr216 led to the proposal that its induction by p53 is part of the mechanism by which inhibitory phosphorylation of MDM2 is attenuated, leading to restoration of p53 levels and reestablishment of the MDM2-p53 feedback loop. This model is complicated, however, by the finding that ser166 is also dephosphorylated by cyclin G1-PP2A physiologically (73). Given that the thr216 and ser166 phosphorylation sites are thought to have opposing effects on MDM2, the biological outcome is not entirely clear. It is possible, however, that this context may be important and under a given set of circumstances cyclin G1 may attenuate MDM2 function but under others favor MDM2 activation. It is also possible that cyclin G1-PP2A-mediated dephosphorylation could be significantly more extensive, because these studies were restricted to the analysis of specific residues, using phospho-specific antibodies or antibodies, the epitopes of which are affected by posttranslational modifications. The development of phospho-specific antibodies to other sites in MDM2 should help resolve this issue.

Further support for cyclin G1 in mediating p53 levels, and for the idea that context may play a pivotal role in the biological outcome of cyclin G1 induction, comes from the development of cyclin G1-null murine embryo fibroblasts. These cells show apparently increased levels of p53 under normal growth conditions. Paradoxically, however, the degree of p53 induction appears to be lower following damage in the G1-nulls within the first 24 h poststress, after which the p53 levels increase significantly in the null cells as compared with the wild-type cells. Strikingly, a normal p53 response could be restored by restoring cyclin G1 expression to the cells ectopically. The explanation for the apparently bizarre behavior of the null cells lies in the ability of cyclin G1 to differentially promote association of MDM2 with ARF during the early part of the response and with PP2A during the late period of the response. Thus, cells lacking cyclin G1 would be less effective in inhibiting MDM2 in the early part of the response, leading to a less pronounced induction of p53, and would fail to recruit PP2A as the response matures, thereby delaying restoration of p53 levels to normal or maintenance of these levels.

Dephosphorylation of the acidic domain of MDM2 is also thought to play a role in the network of events mediating p53 induction. Blattner et al. (62) have shown that key phospho-serine residues in this domain, including serine 240, 242, 260, and 262, become rapidly dephosphorylated in response to ionizing radiation in a manner that clearly precedes p53 accumulation. Mutants of MDM2 with serine to alanine substitutions at these phospho-serine residues, either singly or in pairs, were reported to alleviate, to various degrees, degradation of p53 in keeping with the idea that dephosphorylation of this region is normally required for p53 turnover. Accordingly, stress signals leading to the dephosphorylation of some or all of these residues would attenuate MDM2-mediated p53 degradation and thereby contribute significantly to p53 accumulation. This model would predict that dephosphorylation occurs through stress-dependent inhibition of the activity of a kinase(s) or stimulation of a phosphatase(s), or perhaps both of these events in combination (Fig. 3). At present, however, the enzymes involved in modifying and demodifying this region are not known and will have to be identified before the model can be tested rigorously. It seems unlikely that cyclin G1-PP2A will mediate dephosphorylation of this region because the response to ionizing radiation is very rapid and precedes the p53 induction required to stimulate expression of the cyclin G1.

Conclusions
Growing evidence strongly favors the idea that the MDM2-p53 interaction is the principal target of a variety of stress and survival signals impinging on the p53 pathway. In addition to regulating p53, it is also becoming clear that other targets of MDM2 may be regulated through these same mechanisms. Regulation of MDM2 occurs at several levels including expression of the MDM2 gene, protein-protein interactions, and subcellular localization. It is now clear that extensive posttranslational modification of MDM2 can contribute to these processes as well as regulate MDM2 function directly. The emerging picture is that of a complex activation/stabilization process involving multiple protein modifications, each of which may contribute incrementally to regulating MDM2 function. Most studies, to date, have centered on the response of MDM2 to DNA damage-inducing or hyperproliferative stimuli. However, the p53 pathway is activated by a range of different stresses and it will be important for future studies to address whether these different stimuli impinge on MDM2 through common mechanisms, or perhaps employ novel molecular routes. Given the variety of posttranslational modifications on MDM2, it is entirely possible that these will form part of an integrated series of events that permits a highly sensitive and acutely responsive regulation of MDM2. These may also contribute to MDM2 regulation in a context-dependent fashion, permitting appropriate mechanisms for regulation in a given cell type, or fine-tuning of MDM2 under a given set of circumstances.
Finally, given the important role of MDM2 overexpression, amplification, or activation in the development of many tumors, the ability to inactivate MDM2 function in such tumors would provide a potentially significant approach for therapy. Existing clinical approaches do indeed target the MDM2-p53 interaction through exposure to genotoxic agents, but with the caveat that unwanted genetic changes may occur that allow cancer cells to escape death and become more aggressive. Consequently, therapeutic strategies are currently sought which can induce p53 without causing DNA damage. A comprehensive understanding of the signaling events that govern MDM2 function will pave the way toward developing drugs that can target key signaling enzymes or events in such a manner that permits non-genotoxic clinical manipulation of the p53 response.

In summary, the regulation of MDM2 by posttranslational modification is a highly topical and productive area of research that will undoubtedly deliver some exciting discoveries over the next few years.

References


Posttranslational Modification of MDM2

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