c-Abl Phosphorylates Hdmx and Regulates Its Interaction with p53

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Upon exposure to DNA damage the p53 tumor suppressor is accumulated and activated to stall cellular growth. For this to occur, p53 must be relieved from its major inhibitors, Mdm2 (Hdm2 in humans) and Mdmx (Mdm4; Hdmx in humans). A key mechanism controlling this relief is the post-translational modifications of p53 and its inhibitors. We have previously demonstrated that the stress-activated tyrosine kinase, c-Abl, contributes to the relief of p53 from Hdm2. Because Hdmx is the major inhibitor of p53 activity, the additional possibility that c-Abl protects p53 through targeting Hdmx was explored in this study. c-Abl was found to interact with and to phosphorylate Hdmx. This phosphorylation was enhanced in response to DNA damage. Importantly, we mapped the sites of phosphorylation to the p53 binding domain of Hdmx. One of these phosphorylations, on tyrosine 99, inhibited Hdmx interaction with p53. This inhibition is consistent with the predicted role of this residue in the interaction with p53 based on the crystal structure of the interaction site. Our results show that c-Abl not only targets Hdm2, but also Hdmx, which together contribute to p53 activation in response to DNA damage.

Tight regulation of the p53 protein is critical for tumor suppression, for a proper cellular response to stress, and for mouse development (1). P53 stability and activity are regulated by two major inhibitors, Mdm2 and Mdmx (Mdm4; Hdmx in humans), respectively. The importance of the Mdm genes as p53 regulators was best exemplified by the demonstration that elimination of p53 fully rescues mdm2 and mdm4 null embryos from lethality (2). Mdm2 acts as the direct E3 ligase of p53, promoting it for proteasomal degradation and nuclear export (3). Unlike Mdm2, Mdmx lacks detectable ubiquitin E3 ligase activity and, hence, does not target p53 for degradation but, rather, inhibits p53 transcripcional activity by binding to its transactivation domain (for review, see Ref. 4). Down-regulation or mutations in Mdmx increase the induction of p53 target genes without a change in p53 expression levels (4, 5). The importance of Mdmx as a regulator of p53 is further demonstrated by its link to cancer development. Hdmx is overexpressed or amplified in 10–20% of human cancers including breast, stomach, colon, and lung cancers (for review, see Ref. 6) and in 65% of retinoblastomas (7). Interestingly, in papillary thyroid carcinomas Hdmx expression is down-regulated, although certain Hdmx variants with p53 inhibitory properties were abnormally expressed in these tumors (8). Importantly, overexpression of Mdmx correlates well with a normal status of p53, supporting the notion that this inhibitory pathway is sufficient to suppress p53 and, thus, negates the need for p53 mutation. Thus, abrogating Mdmx function may be an important approach for reactivating p53 in cancer cells (for review, see Ref. 2).

Mdm2 and Mdmx form homo- and heterodimers through their RING domains (9, 10); the ratio of these forms affect their protein stability and the extent of p53 inhibition (4, 11). An important link between the two proteins has been reported under stress conditions. After DNA damage, Mdmx accumulates in the nucleus (11, 12) where it inhibits p53 activity (4, 13), and it is subsequently degraded in an Mdm2-dependent manner (14–16). Mdmx degradation together with the self-degradation of Mdm2 contributes considerably to p53 activation and stabilization upon stress (for review, see Ref. 17).

An important mechanism leading to p53 activation in response to DNA damage involves post-translational modifications, which are mediated by several upstream positive regulators (for review, see Ref. 18). One such activator is the c-Abl non-receptor tyrosine kinase (19). c-Abl has been implicated in the cellular response to stress by promoting cell cycle arrest or apoptosis (20, 21). c-Abl is critical for the accumulation of p53 in response to DNA damage (22). c-Abl activates p53 by neutralizing the inhibitory effects of Hdm2 (22–25). Given the important inhibitory role of Mdmx in p53 regulation and the link between c-Abl and Hdm2, we asked whether c-Abl also protects p53 through targeting Hdmx. We report here a physical and biochemical link between c-Abl and Hdmx. c-Abl interacts with and phosphorylates Hdmx. These phosphorylations are enhanced in response to stress. Tyrosine phosphorylation...
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ations of Hdmx were mapped to the p53 binding domain of Hdmx, and one of these phosphorylations inhibits p53 binding. Overall, our results reveal that Hdmx is a new target of c-Abl, which together with Hdm2 contributes to p53 activation in response to stress.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection Assays, and Cell Treatments—H1299 lung adenocarcinoma cells, lacking p53 expression, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. 293 and 293T human embryonic kidney epithelial cells, MCF-7 breast carcinoma cells, and mdm2/p53 double null (2KO) fibroblasts were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected by the calcium phosphate method as described previously (26). Transfections using polyethyleneimine (PEI; Sigma) were done by mixing DNA with PEI reagent in serum-free medium for 5 min. 1 μl of polyethyleneimine (1 mg/ml) was used for 1 μg of DNA. The polyethyleneimine-DNA complex was applied to the cells for 5–7 h before the medium was replaced. Transfections with FuGENE 6 (Roche Applied Science) were performed in 9-cm dishes according to the manufacturer’s instructions. The FuGENE 6-DNA complex was applied to the cells for 24 h before the medium was replaced. A constant amount of plasmid DNA in each sample was maintained by supplementing with empty plasmid. Usually, cells were transfected with 5–8 μg of the indicated plasmid for a 9-cm plate and with 2–5 μg for a 5-cm plate.

For measuring the cellular response to stress, cells were treated with 3 mM H2O2 (Merck, Germany) together with 1 mM sodium orthovanadate (Sigma) for the indicated times. To induce DNA damage, cells were treated with either 2–2.5 μg/ml doxorubicin hydrochloride (Sigma) or 500 ng/ml neo- carzinostatin (Sigma) in the dark. To inhibit proteasomal degradation, the proteasome inhibitor MG132 (Sigma) was added for 4 h.

Plasmids, in Vitro Site-directed Mutagenesis, and Generation of Lentivirus—Expression plasmids used in this study were human wild-type (wt)2 p53 (pRC-CMV-p53), mouse wt non-tagged and HA-tagged c-abl (pCMV-c-abl, type IV), mouse kinase-defective (KD) c-abl (pCMV-c-ablK290H, type IV) (24), p53-responsive cyclin G-luciferase (26), and an empty vector (pCMV-Neo-BamHI). Hdmx vectors were pcDNA3.1 human HA-hdmx, pcDNA3.1 human wt hdmx, HA-hdmx-C (amino acids (aa) 1–318 followed by 25 alternative aa), hdmx-D (aa 1–206 followed by 13 alternative aa), HA-hdmx-G (Δ27–124 aa). Hdmx deletion mutants were Myc-tagged hdmx ΔN (aa 101–490) and hdmx p53 binding domain (p53 BD)-His-Myc (aa 1–153). The Tyr to Phe substitutions within Hdmx were generated by using the QuikChange site-directed in vitro mutagenesis kit (Stratagene) on the full-length and the p53 BD hdmx forms. For the production of GFP-c-Abl lentivirus, the following vectors were used; GFP-c-Abl was cloned into SIN18.hEF1-WPRE vector using Smal and Xbal sites, pCMVΔR8.91-packaging construct, and pMD2.VSVG envelope construct. For the production of lentivirus these three vectors were co-transfected into HEK293T cells, and lentivirus was collected from the supernatants after 48 h. For the production of si-3′UTR-Hdmx lentivirus (MISSION Library Sigma-Aldrich) the manufacturer’s instructions were followed.

Antibodies—Anti-Hdmx antibodies used were: rabbit polyclonal sera p55, p56 (27), and 13283, mouse monoclonal antibodies 6B1A, 11F4D, and 12G11G (28), and mouse monoclonal MX-82 antibody (Sigma MX-82). Anti-c-Abl antibodies used were the rabbit polyclonal K-12 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the monoclonal Abl-148 antibody (Sigma); the anti-phospho-Hdmx antibodies were phospho-Tyr-55 (Sigma PH-MDMX-55) and phospho-Tyr-99 monoclonal antibodies (Sigma PH-MDMX-169) and anti-phospho-Ser-367 rabbit polyclonal antibody (29). Additional antibodies used were: anti-human p53 monoclonal antibodies PAb1801 and DO1; anti-p53 goat polyclonal antibody (FL-393 Santa Cruz Biotechnology); anti-p53 rabbit polyclonal antibody (FL-393, Santa Cruz Biotechnology); anti-phospho-p53 Ser-20 antibody (Cell Signaling Technology, Inc., Danvers, MA); anti-Hdm2 SMP14 and 2A9 monoclonal antibodies, anti-GFP (Roche Applied Science), anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-14-3-3 tau (BIOSOURCE, Camarillo, CA), horseradish peroxidase-conjugated affinity-purified goat anti-mouse IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA), and EnVision peroxidase anti-mouse or anti-rabbit IgG antibodies (DAKO Corp., Glostrup, Denmark).

Immunoblotting, Immunoprecipitation, in Vivo Kinase Assay and Nickel Pulldown Assay—Western blot analysis and immunoprecipitation assay were performed as previously described (26), whereas the lysis buffer for immunoprecipitation consisted of 50 mM Tris, pH 8.0, 150–300 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 4 mM NaF, and 1 mM sodium orthovanadate. Nickel pulldown and in vivo kinase assay were performed as described previously (24).

Luciferase Assay—This assay was done essentially as previously described (24).

Detection of Phosphorylated Tyr by Mass Spectrometry—Phosphorylated and non-phosphorylated Hdmx proteins were derived from in vivo kinase assay using wt or kinase-defective c-Abl. The proteins were digested using Asp N enzyme, and mass spectrometry was carried out with Qtof2 (Micromass, England) using nanospray attachment (30). Data analysis was done using the biolynx package (Micromass, England), and data base searches were performed with the Mascot package (Matrix Science).

RESULTS

Interaction between c-Abl and Hdmx—To determine whether the protection of p53 by c-Abl involves relief from Hdmx, we examined a possible interaction between c-Abl and Hdmx proteins in cultured cells. HEK293 cells were transfected with expression plasmids for HA-Hdmx either alone or

2 The abbreviations used are: wt, wild type; HA, hemagglutinin; KD, kinase-defective; aa, amino acids; IP, immunoprecipitation; ATM, ataxia telangiec-tasia mutated; GFP, green fluorescent protein; BD, binding domain.

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together with wt or a KD c-AblK290H mutant of c-Abl. Forty-eight hours post-transfection, cell extracts were subjected to immunoprecipitation (IP) using anti-c-Abl Abl-148 antibody followed by immunoblotting (IB) with anti-Hdmx antibodies. As shown in Fig. 1A, Hdmx was efficiently co-precipitated only in the presence of c-Abl, demonstrating an interaction between the two proteins in cultured cells. The interaction between Hdmx and the KD mutant of c-Abl appeared to be weaker. To further establish the interaction between Hdmx and c-Abl, we performed a reversal experiment where IP was done with anti-Hdmx 6B1A antibody and IB with anti-c-Abl antibody. Hdmx efficiently co-precipitated c-Abl (Fig. 1B). These results demonstrate an interaction between the two proteins in cultured cells. Next, we investigated whether endogenous Hdmx interacts with endogenous c-Abl. To answer this question, we performed co-immunoprecipitation experiments using MCF-7 cell line expressing relatively high endogenous Hdmx levels. c-Abl was immunoprecipitated from cell extracts using anti-c-Abl K-12 antibody followed by immunoblotting with anti-Hdmx antibody. As shown in Fig. 1C, Hdmx protein was co-immunoprecipitated by c-Abl, providing evidence for an interaction between the two endogenous proteins.

Both c-Abl and Hdmx interact with p53 and Hdm2 (19). We, therefore, investigated whether the c-Abl/Hdmx interaction is mediated by p53 and/or Hdm2. To address this question, HEK293 cells were transfected with expression plasmids for c-Abl in combination with different Hdmx deletion mutants. First, we tested the involvement of p53 by using Hdmx-G, a product of an alternative splice variant that lacks amino acids 27–124 encompassing the p53 binding domain of Hdmx (Ref. 15 and Fig. 1D). Twenty-four hours post-transfection cell extracts were subjected to immunoprecipitation assay. As shown in Fig. 1E, Hdmx-G (ΔN) bound efficiently to c-Abl, suggesting that p53 is not essential for c-Abl-Hdmx interaction under these conditions. Next we investigated the contribution of Hdm2 to the interaction by using another Hdmx alternative splice variant, Hdmx-D, which lacks the C terminus (amino acids 207–490) including the Hdm2 binding region and contains an additional 13 alternative amino acids after amino acid 206 (Fig. 1F). As shown in Fig. 1F, Hdmx-D (ΔC) efficiently co-precipitated with c-Abl, implying that the Hdm2 binding domain is not critical for c-Abl-Hdmx interaction. To further define the interaction region of c-Abl within Hdmx, a series of additional deletion mutants of Hdmx (Fig. 1D) were tested using the same assay. All the Hdmx deletion mutants tested were able to bind c-Abl. Amino acid composition of the mutants is indicated on the right; note that mutant C and D contains an additional 25 or 13 residues, respectively.

c-Abl Phosphorylates Hdmx within the p53 Binding Domain—Because c-Abl is a tyrosine kinase, we asked whether Hdmx is phosphorylated by c-Abl. There are nine Tyr residues within Hdmx, five of which are clustered within the p53 BD (Fig. 2A) and are conserved among mice, rat, and human (Fig. 2B). In
view of the possible link to p53 regulation, we examined the ability of c-Abl to phosphorylate an Hdmx deletion mutant containing the N-terminal p53 BD (amino acids 1–153) in an in vivo kinase assay. HEK293 cells were transfected with expression plasmids for the Myc- and His-tagged hdmx p53 BD either alone or in combination with wt or c-Abl KD. Twenty-four hours post-transfection Hdmx was isolated from cell extracts using nickel resin, and Tyr phosphorylation of Hdmx was monitored with anti-phosphotyrosine antibody. As shown in Fig. 2C, Tyr phosphorylation of Hdmx p53 BD was detected only in the presence of wt c-Abl, supporting a role for c-Abl in Tyr phosphorylation of Hdmx within the p53 BD.

**Tyrr-99 and Tyrr-55 Are the Major Sites within the p53 BD of Hdmx**—To identify the c-Abl Tyr phosphorylation site(s) within the p53 BD of Hdmx, we employed mass spectrometric analysis along with site-directed mutagenesis. Hdmx p53 BD was phosphorylated in vivo by c-Abl (Fig. 2C) and then subjected to mass spectrometry analysis. Tyrr-55 was identified as a potential Tyr phosphorylation site (data not shown). Additionally, we generated substitution mutants from Tyr(Y) to Phe(F) for each Tyr residue in the p53 BD of Hdmx, either alone or in various combinations. The effect of these substitutions on Hdmx phosphorylation by c-Abl was measured in phosphorylation assays in vivo. HEK293 cells were transfected with expression plasmids for wt hdmx p53 BD or phosphorylation mutants together with wt c-Abl or c-Abl KD. Twenty-four hours post-transfection, Hdmx was precipitated, and Tyr phosphorylation analysis revealed that substitutions of Y99F and Y55F, but not the other Tyr residues within the p53 binding domain, reduced the extent of Hdmx phosphorylation (Fig. 3A and data not shown). Tyr phosphorylation was almost abolished in the Hdmx Y55F,Y99F double mutant (Fig. 3A), supporting the notion that Tyrr-55 and Tyrr-99 are the major c-Abl phosphorylation sites within the p53 BD of Hdmx. Among these two sites, Y99F substitution had a greater effect on Hdmx phosphorylation (Fig. 3A); hence, it was assumed to be a more dominating site. To further verify the phosphorylation of Hdmx on Tyrr-99 and Tyrr-55, anti-phospho- monoclonal antibodies were raised. To determine the specificity of these antibodies, HEK293 cells were transfected with expression plasmids for the Myc and His-tagged hdmx p53 BD in combination with wt c-Abl or c-Abl KD. Specific c-Abl-induced Hdmx phosphorylations on Tyrr-55 and Tyrr-99 were detected with the corresponding anti-phospho antibodies (Fig. 3B). A similar result was obtained when the experiment was repeated with full-length non-tagged wt Hdmx (Fig. 3C).

**Phospho-Tyrr-99 Affects the Phosphorylation of Tyrr-55**—Because the phosphorylation sites reside within the same domain of Hdmx, it was of interest to test a potential dependence between the two sites. To examine this possibility, HEK293 cells were transfected with expression plasmids for Myc and His-tagged wt hdmx p53 BD or phosphorylation mutants either

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**FIGURE 2. c-Abl-dependent tyrosine phosphorylation of Hdmx.** A, schematic representation of the tyrosine residues in Hdmx. The cluster of the tyrosine residues within p53 binding domain of Hdmx is highlighted. B, alignment of the Hdmx amino acid sequence from rat, mouse, and human. The conserved tyrosine residues within Hdmx are highlighted. C, HEK293 cells were transfected with expression plasmids for c-Abl or c-Abl KD either alone or together with His- and Myc-tagged hdmx p53 BD (binding domain; amino acids 1–153). Twenty-four hours post-transfection cells were harvested, and Hdmx p53 BD was isolated from cell extracts using nickel resin. Phosphorylation was detected with anti-phosphotyrosine antibody (4G10), and Hdmx levels were monitored by blotting (IB) with anti-Myc antibody. The expression level of c-Abl was monitored with anti-c-Abl antibody (ABL-148).

**FIGURE 3. c-Abl phosphorylates Hdmx on Tyrr-99 and Tyrr-55 in vivo.** A, HEK293 cells were transfected with the indicated plasmids. Twenty-four hours later Hdmx p53 BD was isolated by nickel resin from cell extracts, and the protein complex was subjected to Western blotting (IB) using the indicated antibodies as described in Fig. 2C. B and C, HEK293 cells were transfected with the indicated plasmids. Twenty-four (B) or forty-eight (C) hours later cell extracts were analyzed by Western blotting using anti-phospho-Tyrr-55 antibody and anti-phospho-Tyrr-99 antibody. Total Hdmx levels were monitored by blotting with anti-Hdmx 1328 polyclonal antibody. The position of Tyrr-55 and Tyrr-99-phosphorylated Hdmx and total Hdmx is indicated. A dividing line in B indicates that lanes were merged from different parts of the same gel.
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Tyr-99 is required for the efficient phosphorylation of Tyr-55, and both tyrosine phosphorylations are independent of Ser-342 and Ser-367 phosphorylation. A–C, HEK293 cells were transfected with the indicated plasmids. Twenty-four (A and B) or forty-eight hours (C) later cell extracts were subjected to Western blotting using either anti-phospho-Tyr-55 antibody (A and C) or anti-phospho-Tyr-99 antibody (A and B). Total Hdmx levels were monitored using anti-Hdmx 1328 polyclonal antibody. The position of Tyr-55 and Tyr-99 phosphorylated Hdmx and total Hdmx is indicated. Hdmx p53 BD mutant was described in Fig. 2C. D, HEK293 cells were transfected with the expression plasmid for c-abl alone or together with Myc and His-tagged hdmx p53 BD either wt or Y99F mutant. Twenty-four hours post-transfection Hdmx was immunoprecipitated using anti-Myc antibody. The level of the co-precipitated c-Abl was monitored by blotting with anti-c-Abl antibody (ABL-148). The expression level of the Hdmx was investigated by using anti-Myc antibody. E, HEK293T cells were transfected with expression vectors for HA-tagged hdmx or hdmx S342A/S367A or empty vector together with c-abl. Cells were either non-treated or treated with 3 μM H2O2 for 45 min together with tyrosine phosphatase inhibitor sodium orthovanadate (Na3VO4; 1 μM) for 5 h before harvest. Twenty-nine hours post-transfection cell extracts were subjected to IP with anti-HA rabbit polyclonal antibody. Immunoprecipitates were analyzed by Western blotting with anti-phospho-Tyr-55-Hdmx, anti-phospho-Tyr-99-Hdmx, and anti-Hdmx MX-82 to monitor total Hdmx levels. To show the absence of Ser-367 phosphorylation of the mutant, the same protein extracts were used in immunoprecipitation with anti-HA monoclonal antibody, and immunoprecipitates were analyzed on Western blot with anti-phospho-Ser-367-Hdmx or anti-Hdmx antibody (BL1258).

A similar effect was observed with full-length non-tagged Hdmx (Fig. 4C). This suggests that phosphorylation on Tyr-99 is required at least partially for an efficient phosphorylation of Tyr-55. An alternative explanation is that the Y99F substitution impairs the interaction between Hdmx and c-Abl. To test this possibility HEK293 cells were transfected with expression plasmids for c-Abl either alone or together with Myc-His-tagged wt or Y99F hdmx p53 BD mutant. Hdmx was immunoprecipitated using anti-Myc antibody, and the amount of co-precipitated c-Abl was determined by IB using anti-c-Abl antibody (Fig. 4D). As shown in Fig. 4D, the Y99F substitution did not reduce the c-Abl-Hdmx interaction but, rather, increased it.

Interplay between c-Abl-mediated phosphorylation of Hdmx and the DNA Damage-induced Hdmx phosphorylation on Ser-342, Ser-367, and Ser-403 and 14-3-3 Binding—It has been shown that Hdmx is independently phosphorylated on Ser-342 and Ser-367, and Ser-403 in response to double strand breaks by ATM (ataxia telangiectasia mutated) or by its target Chk2 (29, 31). Each of these phosphorylations contributes to the increased ubiquitination and degradation of Hdmx after DNA damage, caused by dissociation of the herpes virus-associated ubiquitin-specific protease (HAUSP) from both Hdmx and Hdm2 and promotion of nuclear accumulation of Hdmx (12, 32). Phosphorylation of Ser-342 and Ser-367 was shown to be essential for creating a binding site for several isoforms of the 14-3-3 protein, leading to nuclear localization of Hdmx, and promoting the degradation of Hdmx (29, 33, 34). Because c-Abl is also a target for ATM-dependent phosphorylation after DNA damage, a possible interdependence between the serine and tyrosine phosphorylations on Hdmx was studied.

Initially, we asked whether phosphorylation of Hdmx Tyr-55 and Tyr-99 depends on the phosphorylation of Hdmx-Ser-342 and Ser-367. The extent of c-Abl-activated phosphorylation of Hdmx-Tyr-55 and Hdmx-Tyr-99 was compared between Hdmx S342A/S367A mutant and wild-type Hdmx. We found that the S342A/S367A mutant was still phosphorylated on both Tyr-55 and Tyr-99, indicating that the Tyr-55 and Tyr-99 phosphorylations are independent of Ser-342/Ser-367 phosphorylation (Fig. 4E).

Next, we investigated if phosphorylation of Ser-367 depends on phosphorylation of Hdmx-Tyr-99. To this end, Ser-367 phosphorylation was compared between wild-type Hdmx and Hdmx-Y99F mutant. As can be seen, no significant difference was observed in the Ser-367 phosphorylation between wild-type and Y99F mutant of Hdmx (supplemental Fig. S2A). Another way of approaching this question is to measure whether the Y99F and Y55F substitutions would affect the increased interaction between Hdmx and 14-3-3 proteins upon induction of double strand break by neocarzinostatin. This interaction is dependent on Ser-367 and Ser-342 phosphorylation (29). We found that both Hdmx-Y55F and Y99F mutants were able to bind 14-3-3tau and show increased binding similar

FIGURE 4. Tyr-99 is required for the efficient phosphorylation of Tyr-55, and both tyrosine phosphorylations are independent of Ser-342 and Ser-367 phosphorylation. A–C, HEK293 cells were transfected with the indicated plasmids. Twenty-four (A and B) or forty-eight hours (C) later cell extracts were subjected to Western blotting using either anti-phospho-Tyr-55 antibody (A and C) or anti-phospho-Tyr-99 antibody (A and B). Total Hdmx levels were monitored using anti-Hdmx 1328 polyclonal antibody. The position of Tyr-55 and Tyr-99 phosphorylated Hdmx and total Hdmx is indicated. Hdmx p53 BD mutant was described in Fig. 2C. D, HEK293 cells were transfected with the expression plasmid for c-abl alone or together with Myc and His-tagged hdmx p53 BD either wt or Y99F mutant. Twenty-four hours post-transfection Hdmx was immunoprecipitated using anti-Myc antibody. The level of the co-precipitated c-Abl was monitored by blotting with anti-c-Abl antibody (ABL-148). The expression level of the Hdmx was investigated by using anti-Myc antibody. E, HEK293T cells were transfected with expression vectors for HA-tagged hdmx or hdmx S342A/S367A or empty vector together with c-abl. Cells were either non-treated or treated with 3 μM H2O2 for 45 min together with tyrosine phosphatase inhibitor sodium orthovanadate (Na3VO4; 1 μM) for 5 h before harvest. Twenty-nine hours post-transfection cell extracts were subjected to IP with anti-HA rabbit polyclonal antibody. Immunoprecipitates were analyzed by Western blotting with anti-phospho-Tyr-55-Hdmx, anti-phospho-Tyr-99-Hdmx, and anti-Hdmx MX-82 to monitor total Hdmx levels. To show the absence of Ser-367 phosphorylation of the mutant, the same protein extracts were used in immunoprecipitation with anti-HA monoclonal antibody, and immunoprecipitates were analyzed on Western blot with anti-phospho-Ser-367-Hdmx or anti-Hdmx antibody (BL1258).

alone or in combination with wt c-abl or c-abl KD. The phosphorylation of Tyr-99 was detected in wt Hdmx and the Y55F substitution mutant (Fig. 4A), suggesting that the Tyr-99 phosphorylation is independent of Tyr-55 phosphorylation. Interestingly, the phosphorylation of Tyr-55 was reduced not only in the Y55F mutant but partially also in the Y99F mutant (Fig. 4B).

A similar effect was observed with full-length non-tagged Hdmx (Fig. 4C). This suggests that phosphorylation on Tyr-99 is required at least partially for an efficient phosphorylation of Tyr-55. An alternative explanation is that the Y99F substitution impairs the interaction between Hdmx and c-Abl. To test this possibility HEK293 cells were transfected with expression plasmids for c-Abl either alone or together with Myc-His-tagged wt or Y99F hdmx p53 BD mutant. Hdmx was immunoprecipitated using anti-Myc antibody, and the amount of co-precipitated c-Abl was determined by IB using anti-c-Abl antibody (Fig. 4D). As shown in Fig. 4D, the Y99F substitution did not reduce the c-Abl-Hdmx interaction but, rather, increased it.
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to wt Hdmx after DNA damage, whereas Hdmx-S367A was unable to interact with 14-3-3tau as previously shown (supplemental Fig. S2B). These results are further supported by the preliminary observation that the Y55F and Y99F mutants indeed accumulate in the nucleus, as does wild-type Hdmx, upon treatment of cells with neocarzinostatin (data not shown). Together these result show that phosphorylation of Hdmx-Ser-367 is independent of Hdmx-Tyr-55 and -Tyr-99 phosphorylation.

Phosphorylation of Hdmx on Tyr-99 but Not on Tyr-55 Impairs Its Interaction with p53—The location of Tyr-99 and Tyr-55 within the p53 binding domain of Hdmx begged the question as to whether the phosphorylation of these tyrosines modulates the p53-Hdmx interaction. To address this question, H1299 cells, lacking p53 expression, were transfected with expression plasmids for p53, either alone or together with hdmx p53 BD and c-Abl. Twenty-four hours after transfection, cell extracts were subjected to IP using anti-p53 antibody followed by immunoblotting with either specific anti-phospho-antibodies or anti-Hdmx antibodies. As expected, p53 interacts efficiently with Hdmx. By marked contrast, p53 interaction with the Tyr-99 phosphorylated form of Hdmx was not detected, even after very long exposure of the film (Fig. 5A). This result suggests that Tyr-99 phosphorylation inhibits the interaction of Hdmx with p53. In contrast to Tyr-99, the phosphorylation of Tyr-55 did not impair its interaction with p53 (Fig. 5B) and in some experiments even enhanced it (supplemental Fig. S3). To further establish these findings, we monitored the effect of Hdmx tyrosine phosphorylations at more physiological levels of expression. We have generated MCF-7 stable lines expressing the following full-length HA-tagged Hdmx proteins: wt, Y99F, Y55F, and Y55F,Y99F. To eliminate the effect of endogenous Hdmx on p53 interaction, endogenous Hdmx was downregulated by short hairpin RNA targeting the 3′-untranslated region (data not shown). Using this set of cell lines we examined the interaction between equivalent levels of Hdmx proteins and endogenous p53. p53 was immunoprecipitated (FL-393 antibody), and the amount of bound Hdmx protein was monitored with anti-HA antibody. Our results (Fig. 5C) revealed a slightly enhanced interaction between p53 and Y99F mutant, which is consistent with the above results (panel A). Interestingly, the interaction between p53 and Y55F and especially Y55F,Y99F mutants was reduced. These results strengthen the impact of these phosphorylation sites on the interaction between Hdmx and p53 (see “Discussion”).

Tyr-99 of Hdmx Is Juxtaposed to Pro-27 of p53—Recently, the crystal structure of the N-terminal domain of Mdmx, bound to a short p53 polypeptide, was resolved (35). The analysis of this structure revealed several residues within this region of Mdmx that are important in p53 binding. Strikingly, Tyr-96 in the zebrafish protein, which is equivalent to Tyr-99 in human Mdmx, plays a key role in the interaction between Mdmx and p53. To observe the predicted effect of Tyr-99 phosphorylation on p53 binding, this phosphorylation was modeled on the structure of Mdmx bound to the p53 peptide (residues 15–37). As can be seen in Fig. 6, A and B, the addition of a phosphate group to Tyr-96(Tyr-99) introduces a large hydrophilic entity to the otherwise mostly hydrophobic p53 binding site. This generates a steric clash with proline 27 of p53. The adjacent residues reduce mobility of the phosphotyrosine side chain, making it unlikely that it can change its conformation to allow p53 binding. As shown in a close up view (Fig. 6, C and D) of Tyr-96(Tyr-99)—Pro-27 interaction site, every possible conformation of the phosphate group on Tyr-96(Tyr-99) would clash with Pro-27 of p53. This model strongly supports and explains our results demonstrating an inhibitory effect of Tyr-99 phosphorylation on p53 interaction.

Effect of Stress on Hdmx Phosphorylations by c-Abl—Because c-Abl is activated upon stress, we examined the effect of stress conditions on Tyr-99 phosphorylation as a major c-Abl target site. HEK293 cells were transfected with expression plasmids for wt or Y55F,Y99F hdmx mutant, either alone or in combination with wt c-Abl or KD c-Abl. Cells were either left untreated or treated with a combination of H2O2 with tyrosine phosphatase inhibitor, sodium orthovanadate (Na3VO4), to activate c-Abl and prevent Hdmx dephosphorylation, respectively (36).
Twenty-seven hours post-transfection Hdmx was immunoprecipitated, and protein complexes were analyzed by immunoblotting using anti-phospho-Tyr-99 Hdmx antibody. As shown in Fig. 7A, c-Abl-induced phosphorylation of Tyr-99 increased in response to these stress conditions. The H$_2$O$_2$/Na$_3$VO$_4$ treatment also induced a shift in the electrophoretic mobility of Hdmx. The reason for this shift is not clear, but it has been reported earlier that the DNA damage-induced mobility shift is dependent on phosphorylation of serine 403 (e.g. Ref. 29). To corroborate that the observed stress-induced Hdmx phosphorylation is not cell type-specific, we confirmed these results in p53/mdm2 double KO mouse fibroblasts (2KO 174.2) and in MCF-7 breast cancer cells (supplemental Fig. S4). Furthermore, to examine whether endogenous c-Abl can mediate Hdmx Tyr-99 phosphorylation, HEK293-T cells were transfected with expression plasmid for hdmx. Cells were either left untreated or treated with H$_2$O$_2$/Na$_3$VO$_4$ before being subjected to a phosphorylation assay. As shown in Fig. 7B, Tyr-99 phosphorylation was detected even in the absence of transfected c-Abl but only after H$_2$O$_2$/Na$_3$VO$_4$ treatment to activate endogenous c-Abl. Of note, this phosphorylation was not detected in cells expressing a kinase-defective mutant of c-Abl (Fig. 7A), which is due to the dominant negative effect of this mutant over endogenous c-Abl. The detection of endogenous Hdmx phosphorylation by endogenous c-Abl was unsuccessful, primarily due to the low expression levels of Hdmx after exposure to stress. Our findings of a potential dependence of Tyr-55 phosphorylations on Tyr-99 together with the differential effect of these phospho-

stress conditions. The negative regulation of p53 is governed largely by the Mdm proteins (for review, see Ref. 37). Deregulation of either Mdm proteins can be sufficient to suppress p53, as seen in 10–20% of human cancer cases bearing wt p53 (6). In normal cells the release of p53 from the inhibitory effects of the Mdm proteins is essential for the proper accumulation and activation of p53 (17). This relief involves a spectrum of post-translational modifications of p53 and the Mdm2 proteins (for review, see Refs. 38 and 39). Additionally, recent studies show that Mdmx is also subjected to a spectrum of post-translational modifications (for review, see Ref. 2). These include basal phosphorylation of Mdmx by CDK2/Cdc2(p34) proposed to regulate Mdm2 localization (40) and phosphorylation by CK1-α stimulating the Mdmx-p53 interaction (41). Additionally, Akt1 mediates Hdmx phosphorylation, leading to Hdmx stabilization and induced 14-3-3 binding (42). Upon stress, Mdmx is modified by key regulators of biological response to DNA damage like checkpoint kinases 1 and 2 (Chk1, Chk2) and ATM (for review, see Refs. 2 and 43). Furthermore, ATM-dependent phosphorylations of Mdmx are required for the efficient damage-induced Mdmx degradation, at least partially by affecting binding to herpes virus-associated ubiquitin-specific protease (HAUSP) (44), and also by stimulating the interaction with 14-3-3 proteins, which contributes to nuclear accumulation and degradation of Hdmx under DNA-damage conditions (29, 31). The degradation of Hdmx in response to stress is critical for p53 activation (for review, see Ref. 17). An additional important arm of the ATM
c-Abl Regulates Hdmx-p53 Interaction

A

\[
\begin{align*}
\text{Hdmx wt} & \quad + \quad H_2O_2/Na_3VO_4 \\
\text{Hdmx Y55,99F} & \quad + \quad + \quad + \\
\text{c-Abl} & \quad wt \quad KD \quad wt \quad wt \quad wt \quad wt \quad KD \quad wt \quad KD \\
\text{IP} & : \text{anti-Hdmx} \\
\text{IB} & : \text{anti-phospho-H99 Hdmx} \\
\end{align*}
\]

B

\[
\begin{align*}
\text{Hdmx} & \quad + \quad + \\
\text{H}_2O_2/\text{Na}_3\text{VO}_4 & \quad + \\
\text{IP} & : \text{anti-Hdmx} \\
\text{IB} & : \text{anti-phospho-H99 Hdmx} \\
\end{align*}
\]

C

Time course for exposure to doxorubicin (1 hour)

| Time before harvesting (hours) | 0 | 2 | 4.5 | 6 | 6.5 | 8.5 | 10.5 |
|-------------------------------|---|---|-----|---|-----|-----|------|
| Pull down Ni beads IB         |   |   |     |   |     |     |      |
| Pull down Hdmx IB            |   |   |     |   |     |     |      |
| Pull down Hdmx p53           |   |   |     |   |     |     |      |
| IB: anti-phospho-S20 p53     |   |   |     |   |     |     |      |
| IB: anti-Hdmx                |   |   |     |   |     |     |      |

FIGURE 7. Stress induces Hdmx phosphorylations by c-Abl. HEK293 (A) or HEK293-T cells (B) were transfected with the indicated plasmids. Cells were either left untreated or were treated with 3 mM H_2O_2, together with tyrosine phosphate inhibitor sodium orthovanadate (Na_3VO_4, 1 mM) for 20 min before harvest. Twenty-four hours post-transfection Hdmx was immunoprecipitated from cell extracts using anti-Hdmx antibody, 6B1A antibody was used in A, and a mixture of rabbit polyclonal anti-Hdmx antibodies (p55, p56, and 1328) was used in B. Hdmx phosphorylated on Tyr-99 was detected with anti-phospho-Tyr-99 antibody, and total Hdmx level was monitored with 6B1A (A) or MX-82 (B) anti-Hdmx antibody. C, HEK293 cells were infected with c-Abl-GFP lentivirus. Seventy-two hours later cells were transfected with His and Myc-tagged hdmx p53 BD plasmid. Forty-eight hours post-transfection, cells were either left untreated or were treated at different time points with doxorubicin 2.5 μg/ml for 1 h. All the cells were harvested at the same time point. The time course of the experiment is shown in the upper panel, Hdmx p53 BD was isolated from cell extracts as described in Fig. 2C, and the protein complex was subjected to Western blotting using either anti-phospho-Tyr-55 antibody or anti-phospho-Tyr-99 antibody. Total Hdmx levels were monitored by blotting with anti-Hdmx antibody (MX-82). Stress-induced activation of p53 was followed by using anti-phospho-Ser20 antibody.

stress response is the activation of c-Abl. We have previously shown that p53 activation by c-Abl requires the neutralization of the inhibitory effects of Mdm2 (22, 23). c-Abl interacts with and phosphorylates Hdm2, thereby impairing its E3 ligase activity toward p53 (24, 25). In addition, c-Abl stabilizes the interaction of p53 tetramer with certain promoters, such as p21 (45, 46).

In this study we explored a new pathway by which c-Abl regulates p53. We found that c-Abl interacts with Hdmx and phosphorylates it in cultured cells. Specifically, we identified Tyr-55 and Tyr-99 as c-Abl phosphorylation sites. These phosphorylations are independent of Hdmx phosphorylation by ATM or Chk2, and they do not affect 14-3-3/Hdmx binding. Importantly, however, both tyrosines reside within the p53 binding region. We found that phosphorylation of Tyr-99 inhibits the interaction between Hdmx and p53 (Fig. 5A). This finding is highly supported by a recent study describing the crystal structure of the N terminus of Mdmx bound to a p53 peptide. This study highlighted Y96 (humanized zebrafish Mdmx equivalent to Tyr-99) as a critical residue for p53 binding (35). The addition of phosphorylated Tyr-99 to the modeled structure reveals the expected inhibition of p53 binding (Fig. 6). Because a physical interaction is required for the inhibition of p53 by Hdmx (47), interference with this interaction provides an efficient mechanism by which to modulate the Hdmx-p53 regulation. Our results imply that in response to stress, c-Abl protects p53 also by modulating the Hdmx-p53 interaction through Tyr-99 phosphorylation. This defines the first phosphorylation of Hdmx, which inhibits its interaction with p53. Consequently, it is predicted that this phosphorylation would impair the inhibitory effect of Hdmx on p53. To test this prediction we measured the effect of Y99F substitution on the inhibitory effect of Hdmx on p53 transcriptional activity using a luciferase reporter assay. Although the substitution mutant consistently inhibited more efficiently than wt Hdmx, the effect was very modest (supplemental Fig. S5). Why is this effect so small? First, we assume that only a fraction of Hdmx molecules undergo tyrosine phosphorylation, to which the phosphorylation mutant is compared. Second, it needs to be stressed that in this type of experiments c-Abl is an efficient activator of p53 (22–24). It is, therefore, difficult to achieve an optimal stoichiometry between Hdmx, c-Abl, and p53 that distinguishes the direct effect of c-Abl on p53 from the indirect effect via the Mdm proteins.

The phosphorylation of Hdmx on Tyr-55 did not impair its interaction with p53, and in some experiments it even seemed to enhance this interaction (supplemental Fig. S3). Moreover, interaction of endogenous p53 with Y55F and especially with Y55F,Y99F Hdmx was reduced as compared with wt and Y99F Hdmx (Fig. 5C), emphasizing the role of Tyr-55 in the Hdmx-p53 interaction. Thus, c-Abl phosphorylates two tyrosines within the p53 binding region of Hdmx, which differently affect its interaction with p53. How could this observation be reconciled? In an attempt to understand this observation, we monitored the pattern of these phosphorylations in response to DNA damage, a stress signal known to activate c-Abl and p53. Interestingly, phosphorylations are independent of Hdmx phosphorylation by ATM or Chk2, and they do not affect 14-3-3/Hdmx binding.

The phosphorylation of both sites was enhanced in response to DNA damage, the pattern of phosphorylation of the two sites differed. The extent of Tyr-99 phosphorylation increased moderately, with a possible temporary reduction at around 8–9 h after damage. On the other hand, Tyr-55 phosphorylation appeared with a short delay after Tyr-99 phosphorylation but continued to increase stronger with time after...
exposure (Fig. 7C and data not shown). Furthermore, Tyr-55 phosphorylation was impaired in the absence of Tyr-99, suggesting a potential sequential phosphorylation where Tyr-99 precedes Tyr-55 (Fig. 4). On the basis of these findings we propose a scenario whereby Tyr-99 phosphorylation occurs early after exposure to DNA damage, preventing Hdmx-p53 interaction, thereby contributing to p53 activation. Subsequent phosphorylation of Hdmx on Tyr-55 may counteract the effect of Tyr-99 on p53 interaction, thereby contributing to the recovery of p53 from stress-induced activation. Overall, we show here that c-Abl not only targets Hdm2, but also targets Hdmx, which contributes to its role in p53 regulation.

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