The Prolyl Isomerase Pin1 Affects Che-1 Stability in Response to Apoptotic DNA Damage*

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Francesca De Nicola4,5, Tiziana Bruno4, Simona Iezzi5,1, Monica Di Padova4,5, Aristide Floridi4,5, Claudio Passananti4, Giannino Del Sal**, and Maurizio Fanciulli**2

From 1Laboratory “B”, Experimental Research Center and 2Rome Oncogenicomic Center, Regina Elena Cancer Institute, Via delle Messi d’Oro 156, 00158 Rome, Italy, 3Department of Experimental Medicine, Via Vetoio, Coppito 2, University of L’Aquila, 67100 L’Aquila, Italy, 4Istituto di Biologia e Patologia Molecolari, Consiglio Nazionale delle Ricerche, Viale Marx 43, 00137 Rome, Italy, and **Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, Area Science Park, and Dipartimento di Biochimica Biofisica e Chimica delle Macromolecole, Universita’ di Trieste, 34100 Trieste, Italy

We have previously demonstrated that DNA damage leads to stabilization and accumulation of Che-1, an RNA polymerase II-binding protein that plays an important role in transcriptional activation of p53 and in maintenance of the G2/M checkpoint. Here we show that Che-1 is down-regulated during the apoptotic process. We found that the E3 ligase HDM2 physically and functionally interacts with Che-1 and promotes its degradation via the ubiquitin-dependent proteasomal system. Furthermore, we found that in response to apoptotic stimuli Che-1 interacts with the peptidyl-prolyl isomerase Pin1 and that conformational changes generated by Pin1 are required for Che-1/ HDM2 interaction. Notably, a Che-1 mutant lacking the capacity to bind Pin1 exhibits an increased half-life and this correlates with a diminished apoptosis in response to genotoxic stress. Our results establish Che-1 as a new Pin1 and HDM2 target and confirm its important role in the cellular response to DNA damage.

Che-1 is a human RNA polymerase II-binding protein that is highly conserved from yeast to man and involved in the regulation of gene transcription (1–5). Che-1 interacts with Rb and affects its growth-suppressing activity by interfering with Rb-mediated recruitment of histone deacetylase I onto the promoters of E2F1-responsive genes (1, 6). The rat ortholog of Che-1, AATF, directly interacts with nuclear hormone receptors and enhances nuclear hormone receptor-mediated transactivation in a hormone-dependent way (7, 8). In addition, the mouse ortholog of Che-1, Traube, is essential for proliferation during early embryogenesis (2). Despite the role of Che-1/Traube/ AATF in proliferation, this protein is also involved in the regulation of apoptosis, exhibiting strong anti-apoptosis activity (4, 9). In agreement with this, Che-1 expression is down-regulated in several tumors and in particular in ~80% of colon carcinomas examined (10) and Che-1 overexpression induces cell cycle arrest in human colon carcinoma cell lines through induction of the cyclin-dependent kinase inhibitor p21Waf1/p21 (10).

Recently, we have shown that DNA damage induced by different genotoxic agents associates with Che-1 phosphorylation and extended half-life (11). These post-translational modifications are induced by ataxia telangiectasia mutated (ATM)3 and Chk2, both of which phosphorylate Che-1 on specific residues and are functionally linked to the DNA damage-induced G2/M checkpoint. Moreover, microarray and chromatin immunoprecipitation analyses show that Che-1 is involved in p53 transcription and consequently of several p53 target genes, identifying a new pathway by which ATM and Chk2 can modulate p53 levels (11).

The peptidyl-prolyl isomerase Pin1 has recently been identified as an important regulator of cell proliferation and the DNA replication checkpoint (12–16). Pin1 specifically recognizes phospho-serine or -threonine followed by proline (pS/pT-P) catalyzing conformational changes in its substrates (12, 17), which have profound effects on activity, stability, and subcellular localization (18–27). Although Pin1 is overexpressed in several human tumors (25) and its depletion induces apoptosis (12), it has also been demonstrated that Pin1 is involved in stabilizing both p53 and p73 proteins and in the regulation of their apoptotic functions (28–31). DNA damage enhances the interaction between Pin1 and p53 (28–30) and Pin1 mediates conformational changes in p53, affecting its capacity to interact with HDM2 (29). HDM2 (originally identified in mice as the murine double minute 2 protein, MDM2) acts as a ubiquitin E3 ligase, transferring ubiquitin to p53, promoting its degradation via the ubiquitin-dependent proteasomal system (31–33).

Here we report that Che-1 levels are down-regulated in response to apoptosis induced by HDM2/MDM2-mediated ubiquitin-dependent degradation. We demonstrate that Che-1 interacts with Pin1 following apoptotic DNA damage and that conformational changes, driven by Pin1, are required for Che-1/HDM2 interaction and Che-1 degradation. Furthermore, we show that a Che-1 mutant that does not interact with Pin1 is more stable and exhibits greater anti-apoptotic activity.

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1 Recipient of a Fondazione Italiane per la Ricerca sul Cancro fellowship.
2 To whom correspondence should be addressed. Tel.: 3906-52665566; Fax: 3906-52662578; E-mail: fanciulli@ifo.it.
3 The abbreviations used are: ATM, ataxia telangiectasia mutated; E3, ubiquitin-protein isopeptide ligase; GST, glutathione S-transferase; HA, hemagglutinin; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA; Dox, doxorubicin; MEF, mouse embry fibroblast.
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MATERIALS AND METHODS

Cell Culture, Transfections, and Analysis—HCT116 human colon carcinoma cell line, wild-type, and MDM2/p53 null mouse embryo fibroblasts (kindly provided by Dr. G. Lozano, University of Texas, Houston) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfections were carried out by calcium phosphate precipitation as described (1) or by Lipofectamine 2000 (Invitrogen). Transfection efficiency ranged from 30 to 50%. Flow cytometric analyses were performed as previously described (11). Caspase 3/7 activities were measured using the Caspase-Glo 3/7 kit (Promega) following the manufacturer’s instructions. Doxorubicin was purchased from Sigma, and MG132 was purchased from Calbiochem and used at 20 μM.

Clinical grade DDP (Cisplatin Teva) was obtained from Teva Pharma. Calf phosphatase was purchased from New England Biolabs and used as previously described (29).

Recombinant Plasmids and Proteins—Myc-tagged Che-1 and its partial deletion mammalian expression vectors have been previously described (1). Glutathione S-transferase (GST) -tagged Pin1 protein was produced as previously described (29). The expression vector containing MDM2 wild type and MDM2 ΔRing cDNA were kind gifts from Dr. Levine and Dr. Oren, respectively. The other MDM2 deletion mutants were produced in Dr. Moretti’s laboratory. Pull-down experiments were performed as previously described (1). Mutagenesis of Myc-Che-1 was performed using the QuikChange mutagenesis system (Stratagene) following the manufacturer’s instructions.

Antibodies—The following rabbit polyclonal antibodies were used: anti-Che-1 (1), anti-Pin1 (29), anti-p53 (sc-6243; Santa Cruz Biotechnology), anti-cleaved caspase-3 (Cell Signaling). Mouse monoclonal antibodies anti-Myc 9e10 (Invitrogen), -FLAG M2 (Sigma), -α-tubulin and –HA probe (sc-8035 and sc-7392; Santa Cruz Biotechnology), -HDM2/MDM2 (a gift from Dr. Moretti, Consiglio Nazionale delle Ricerche, Rome), and -Parp (BD Biosciences) were used.

Immunoprecipitations and Western Blot Analysis—For immunoprecipitation experiments cells were lysed by incubation at 4 °C for 30 min in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM NaF, 3 mM Na3VO4, 10 mM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin). After high speed centrifugation, the lysates were precleared with 20 μl of protein A/protein G beads (Santa Cruz Biotechnology) and immunoprecipitated by standard procedures. Western blots were performed by standard procedures, and immunoreactivity was detected by ECL chemoluminescence reaction (Amersham Biosciences).

RT-PCR Analysis—For semi-quantitative reverse transcription PCR (RT-PCR) analysis total RNA isolated using TRizol reagent (Invitrogen) was subjected to RT-PCR using a Platinum quantitative RT-PCR kit (Invitrogen) according to the manufacturer’s instructions. PCR products were separated by 2% agarose gel electrophoresis. PCR conditions were as follows: one cycle at 95 °C for 5 min, followed by 25 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The primers employed in RT-PCR analysis were as follows: Che-1 Forward primer CCGCTCGAGATGCCATTCTGAGTGT.

RESULTS

Che-1 Is Degraded during Apoptosis—We had previously shown that, in response to genotoxic stresses, the checkpoint kinases ATM/ATR and Chk2 physically and functionally interact with Che-1 and promote its phosphorylation and stabilization (11). Furthermore, we have found that Che-1-mediated transcriptional regulation is required for DNA damage-induced, p53- and p21-mediated maintenance of the G1/M checkpoint (11). Moreover, AATF, the rat ortholog of Che-1, exhibits anti-apoptotic activity by interfering with Dlk kinase (4). On the basis of these results, we investigated the effect of apoptosis on the Che-1 protein levels. To this end, HCT116 human colon carcinoma cells were treated with a lethal dose (1 μM) of the anti-tumor drug doxorubicin (Dox) for different times. As is shown in Fig. 1A, Che-1 protein levels accumulated following 2 h of Dox treatment, preceding p53 stabilization, whereas protein levels decreased concomitantly with the apoptotic process (Figs. 1A and B). Similar results were observed when HCT116 cells were treated with cis-diammine-dichloroplatinum (cisplatin) (Fig. 1C).

To identify the mechanism(s) of Che-1 down-regulation, Che-1 mRNA levels in response to DNA damage were assessed by RT-PCR. Che-1 expression was not altered either by growth arrest or apoptotic Dox treatments (Fig. 1D), confirming previous results showing post-translational regulation of Che-1 in response to genotoxic stress (11). Consistent with this, Che-1 promoter activity, assessed by a luciferase-reporter gene did not show appreciable differences upon drug treatment (data not shown). Thus, repression of Che-1 must occur, at least in part, through its degradation. Ubiquitin-dependent proteasomal degradation represents a major pathway for regulated protein levels in eukaryotic cells; we therefore treated HCT116 cells transfected with an HA-tagged ubiquitin plasmid for different times with Dox. Che-1 immunoprecipitation from treated cells exhibited incremental ubiquitination during the induction of apoptosis (Fig. 1E). Taken together, these data confirm that Che-1 levels are reduced by proteasome-mediated degradation and that following apoptotic induction this process is enhanced.

HDM2/MDM2 Binds Che-1 in Response to DNA Damage—Protein ubiquitination results from a multienzymatic pathway responsible for the transfer of ubiquitin onto a specific target protein mediated by an E3 ubiquitin ligase. Polyubiquitinated proteins are subsequently recognized and hydrolyzed by the 26 S proteasome (34). An E3 ligase implicated in the regulation of the response to DNA damage is HMD2/MDM2, which promotes p53 ubiquitination and degradation (31, 32). We there-
fore speculated that HDM2 may also interact with and regulate Che-1 degradation. Indeed, strong interaction between Che-1 and HDM2 was detected following DNA damage-induced apoptosis (Fig. 2A), indicating that both proteins belong to the same complex and that apoptosis induces the Che-1 presence in this complex. To characterize the regions involved in this interaction, we performed co-immunoprecipitations from HCT116 cells transfected with partial deletions of Che-1 or MDM2 and treated with Dox. As is shown in Fig. 2B, the Che-1 region spanning amino acids 270–370 is necessary to bind HDM2, whereas the first 50 amino acids of HDM2 are required for HDM2/Che-1 interaction (Fig. 2C). Notably, this region is also involved in p53/MDM2 interaction (35). Next, we tested HDM2/Che-1 interaction during Dox treatment. As shown in Fig. 2D, Che-1 strongly bound HDM2 after 12 h of Dox treatment and this interaction was still barely detectable after 24 h of treatment.

**HDM2/MDM2 Is a Negative Regulator of Che-1 Protein Levels**—We evaluated whether HMD2/MDM2 is involved in the degradation of Che-1. For this purpose, mouse embryo fibroblasts (MEFs) double knock-out (Mdm2+/− and p53+/−) mice were transfected with MDM2 wild type or with a mutant ΔRing lacking the enzymatic activity and HA-tagged ubiquitin plasmid (Fig. 3A). Che-1 immunoprecipitation exhibited a strong increase of ubiquitination in cells transfected with MDM2 but not in cells transfected with the ΔRing mutant (Fig. 3A), thus indicating that Che-1 is a direct target of E3 ligase activity of MDM2. Similar results were obtained when MEF cells from wild-type (Mdm2+/+) and double knock-out (Mdm2+/− and p53−/−) mice were treated with Dox for different times. As is shown in Fig. 3B, in wild-type MEFs Che-1 levels were low and almost undetectable following 12 h of drug treatment. In contrast, MEFs lacking MDM2 expression exhibited higher levels of Che-1 protein, which was less degraded during apoptosis (Fig. 3C). Consistent with
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FIGURE 3. HDM2/HMDM2 is a negative regulator of Che-1 protein levels. A, MDM2−/− MEF cells were transfected with HA-ubiquitin and MDM2. MDM2 ΔRing, or empty vector. Cell extracts were immunoprecipitated with anti-Che-1 polyclonal antibody and analyzed by Western blot using the indicated antibodies. B, Western blot analysis of mouse embryo fibroblasts (MEF) wild type or MDM2−/− treated with 1 μM Dox for the indicated times and probed with the indicated antibodies. C, Western blot analysis of MDM2−/− mouse embryo fibroblasts (MEF) transfected with the indicated vectors and treated with 1 μM Dox for the indicated times. D, MDM2−/− MEF cells were transfected with HA-ubiquitin and MDM2 or empty vector and treated with 1 μM Dox for the indicated times. Cell extracts were immunoprecipitated with anti-Che-1 polyclonal antibody and analyzed by Western blot using the indicated antibodies.

FIGURE 4. Pin1 interacts with Che-1 and affects its stability. A, in vitro binding analysis of GST-Pin1 fusion protein with cell extracts from HCT116 cells treated with or without 1 μM Dox. The membrane was analyzed by Western blot using anti-Che-1 antibody. B, Western blot analysis of HCT116 cells incubated with or without 1 μM Dox. Cell lysates were incubated for 30 min with or without Calf intestinal alkaline phosphatase (CIAP), immunoprecipitated with anti-Che-1 antibody, and immunoblotted with the indicated antibodies. C, HCT116 cells were transiently transfected with siRNA smartPool control (siControl) or siRNA Pin1 (siPin1) and 72 h later treated with 1 μM Dox. Cell extracts were immunoprecipitated with anti-HDM2 antibody and immunoblotted with the indicated antibodies. D, Western blot analysis of HCT116 cells transiently transfected with siRNA smartPool control (siControl) or siRNA Pin1 (siPin1) and 72 h later treated with 1 μM Dox for the indicated times. Cell extracts were immunoblotted with the indicated antibodies. E, Western blot analysis using the indicated antibodies of HCT116 cells transfected with HA-ubiquitin, siRNA smartPool control (siControl), or siRNA Pin1 (siPin1) and 72 h later treated with 1 μM Dox for 12 h. Cell extracts were immunoprecipitated with anti-Che-1 polyclonal antibody.

these findings, transfection of MDM2 into Mdm2−/− and p53−/− cells increased Che-1 polyubiquitination (Fig. 3D). Together, these data indicate that HDM2/HMDM2 directly ubiquitines Che-1 and in such way regulates Che-1 stability during apoptosis.

Pin1 Interacts with Che-1 and Affects Its Stability—The finding that the interaction between Che-1 and HDM2 is strongly increased by apoptotic induction prompted us to investigate the possible mechanism/s by which this interaction is regulated. Previous evidence has indicated that Pin1-mediated conformational changes in phosphorylated p53 influence its capacity to interact with MDM2 (29). Because we have demonstrated that in response to DNA damage Che-1 is phosphorylated at least in part by ATM/ATR and Chk2 (11), we hypothesized that Pin1 may also interact with Che-1. To test this, we used GST-Pin1 in pull-down assays. Pin1 bound to Che-1 only following Dox treatment (Fig. 4A). Consistent with this result, co-immunoprecipitation using anti-Che-1 antibody and extracts from untreated and Dox-treated cells confirmed Che-1/Pin1 interaction in DNA-damaged cells (Fig. 4B). Moreover, this interaction requires Che-1 phosphorylation, as demonstrated by the lack of binding when Che-1 was dephosphorylated by phosphatase treatment (Fig. 4B). We also evaluated whether Pin1 could affect Che-1/HDM2 interaction by coimmunoprecipitation with anti-HDM2 antibody in HCT116 cells, in which Pin1 expression was depleted by siRNA, treated with Dox. As shown in Fig. 4C, siRNA inhibition of Pin1 strongly reduced the amount of Che-1 bound to HDM2, indicating that Pin1-mediated Che-1 conformational changes are required for its interaction with HDM2. Consistent with this, Pin1 depletion in HCT116 cells produced an increase in Che-1 stability (Fig. 4D) and a decrease of its ubiquitination (Fig. 4E). These data demonstrate that in response to apoptotic stimuli, Pin1 binds Che-1 and affects its stability by increasing its capacity to interact with HDM2.

Pin1 binds phosphoproteins by recognizing pSer/Thr-Pro motifs via its amino-terminal WW domain (23, 37). One such putative consensus site is present in Che-1 at residues Thr-144 (Fig. 5A). To evaluate its potential involvement in Pin1/Che-1 interaction, a Myc-tagged Che-1 mutant with Thr-144 substituted with alanine (AP) was assayed in GST-Pin1 pull-down assays. As shown in Fig. 5B, the AP mutant failed to bind GST-Pin1, demonstrating that Thr-144 is required for this interaction. Next, we assessed whether the AP mutant was able to interact with MDM2. Co-immunoprecipitation experiments performed with HCT116 cells transfected with wild-type Myc-Che-1

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and AP mutant and treated with Dox indicated that Myc-Che-1, but not AP, strongly interacted with MDM2 (Fig. 5C). Consistent with this, the stability of the AP mutant was not affected by Dox treatment (Fig. 5D), supporting the possibility that Pin1 regulates Che-1/MDM2 interaction and Che-1 stabilization.

**Apoptosis Requires Che-1 Degradation**—Given that apoptosis induces Che-1 degradation through its modification, we examined the effects of wild-type Che-1 or AP mutant overexpression on apoptosis induced by Dox. Overexpression of wild-type Che-1 significantly reduced the number of apoptotic cells following Dox treatment, with the AP mutant exhibiting a greater effect (Fig. 6A). Similar results were obtained upon measuring the effect of Che-1 upon caspase 3/7 activities (Fig. 6B), thus implying that phosphorylation and ubiquitination of Che-1 are essential for induction of apoptosis. Consistent with these findings, a time course treatment with DPP confirmed that Che-1 exerts a strong anti-apoptotic activity (Fig. 6C).

**DISCUSSION**

Proteins that regulate the cell cycle are themselves regulated by transcriptional and posttranslational mechanisms, including phosphorylation, acetylation, and ubiquitination, which can also alter their functions. We have previously shown that in response to genotoxic stresses the transcriptional regulator Che-1 is phosphorylated and stabilized by ATM and Chk2 kinases (11). In particular, we found that the half-life of Che-1 is tightly regulated by the proteasome and that specific phosphorylations protect the protein from degradation. Moreover, we have shown that Che-1 is required for maintenance of the G2/M checkpoint and that its down-regulation by siRNA sensitizes cancer cells to chemotherapy (11).

In this study, we demonstrate that in response to apoptotic stimuli HDM2/MDM2 protein negatively regulates Che-1 by promoting its ubiquitin-mediated degradation. We show that Che-1 is a direct target of HDM2/MDM2 and that this interaction is increased following induction of apoptosis. We also report that HDM2/MDM2-Che-1 interaction is regulated by Pin1, which modifies Che-1 structure, increasing its capacity to interact with HDM2/MDM2. Indeed, Pin1 bound Che-1 only upon Dox-mediated apoptosis, and siRNA-mediated Pin1 depletion strongly decreased HDM2/MDM2-Che-1 interaction, resulting in Che-1 stabilization. Pin1 regulates several cellular processes (12–16) by binding and isomerizing prolyl bonds within phospho-Ser/Thr-Pro motifs. The effect of Pin1 on Che-1 was phosphorylation-dependent, with a lack of interaction observed upon Che-1 dephosphorylation. Mutation of

**FIGURE 5. Che-1 Thr-144 is a Pin1 target.** A, amino acid sequence of Che-1. The putative binding site of Pin1 is circled and in red. B, in vitro binding analysis of GST-Pin1 fusion protein with lysates from HCT116 cells transfected with Myc-Che-1 or with Che-1 AP mutant and treated with or without 1 μM Dox. Western blot analysis was performed using anti-Myc monoclonal antibody. C, HCT116 cells were transfected and treated as in panel B. Cell extracts were immunoprecipitated with anti-HDM2 (left) or anti-Myc (right) antibodies and immunoblotted with the indicated antibodies. D, Western blot analysis of HCT116 cells transfected as in panel B and treated with 1 μM Dox for the indicated times. Cell extracts were immunoblotted with the indicated antibodies.
the single Ser/Thr-Pro motif (Thr-144) in human Che-1 reduced Che-1 capacity to bind both Pin1 and HDM2 (Fig. 5, B and C). In agreement with this, the AP mutant half-life was not affected by Dox-mediated apoptosis, confirming that Pin1 regulates the degradation of Che-1. In addition, the observation that Che-1 is degraded during the induction of apoptosis even in the absence of MDM2 (Fig. 3B) leads us to hypothesize that Che-1 is a substrate of other E3 ligase enzymes.

In agreement with previous findings (4, 11), we confirm that Che-1 is an anti-apoptotic factor and that its degradation is required for executing the apoptotic program. Indeed, overexpression of Che-1 AP mutant counteracted apoptosis more efficiently than wild-type protein. Therefore, it possible to speculate that Pin1 is required for the apoptotic response to DNA damage, not only stabilizing p53 and p73 (28–31) but also increasing Che-1 degradation.

**FIGURE 6. Apoptosis requires Che-1 degradation.** A and B, HCT116 cells were transfected with Myc-Che-1 or with Che-1 AP mutant and treated with 1 μM Dox. 48 h later, cell death (A) or caspases 3/7 (B) activities were assayed. C, HCT116 cells were transfected with Myc-Che-1 or with Che-1 AP mutant and treated with 5 μg/ml cis-diammine-dichloroplatinum (cisplatin). 48, 72, and 96 h later, cell death was assayed. Data are presented as the mean ± S.D. from three independent experiments performed in duplicate.

**FIGURE 7. Model to explain Che-1 activation in response to DNA damage.** Non-apoptotic genotoxic stresses induce Che-1 stabilization through its phosphorylation by ATM and Chk2. Stabilization of Che-1 produces an increase of p53 and p21 expression and contributes to arrest cell growth in response to DNA damage. Apoptotic DNA damage induces Che-1 Thr-144 phosphorylation and Che-1/Pin1 interaction. Pin1 catalyzes Che-1 conformational changes, increasing Che-1/HDM2 interaction and in such way Che-1 degradation.
Recently, association between Che-1 and TSG101 has been reported (8). TSG101 is a tumor suppressor that stabilizes proteins, including MDM2 and p21, by inhibiting their polyubiquitination (36, 38). Indeed, TSG101 contains an inactive Ubc domain and may act as a dominant-negative inhibitor of ubiquitination (37). Che-1/TSG101 interaction could protect Che-1 from polyubiquitination and subsequent degradation. Conversely, Che-1 modifications induced by Pin1 in response to apoptotic stimuli could inhibit Che-1/TSG101 interaction, permitting Che-1/HDM2 binding and Che-1 degradation.

Computational analysis has identified the Thr-144 residue within Che1 as a potential target for kinases, including p38 mitogen-activated protein kinase or cdk5. Furthermore, preliminary evidence indicates that Che-1 is a substrate of the pro-apoptotic kinase Hipk2 (data not shown). The identification of the kinase(s) responsible for Thr-144 phosphorylation in Che-1 should provide new insight into the intracellular signaling pathway involved in the regulation of Che-1.

In conclusion, our data allow us to hypothesize a model in which different stresses activate Che-1 to increase its stability in case of growth arrest or to promote its degradation driving apoptosis (Fig. 7).

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