Quantitative Proteomic Analysis of Myc-induced Apoptosis

A DIRECT ROLE FOR Myc INDUCTION OF THE MITOCHONDRIAL CHLORIDE ION CHANNEL, mtCLIC/CLIC4

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Myc is a key regulatory protein in higher eukaryotes controlling important cellular functions such as proliferation, differentiation, and apoptosis. Myc is profoundly involved in the genesis of many human and animal cancers, and the abrogation of Myc-induced apoptosis is a critical event in cancer progression. Because the mechanisms that mediate Myc-induced apoptosis are largely unknown, we analyzed protein expression during Myc-induced apoptosis using an isotope-coded affinity tag quantitative proteomics approach and identified that a proapoptotic mitochondrial chloride ion channel, mtCLIC/CLIC4, is induced by Myc. Myc binds to the mtCLIC gene promoter and activates its transcription. Suppression of mtCLIC expression by RNA interference inhibited Myc-induced apoptosis in response to different stress conditions and abolished the cooperative induction of apoptosis by Myc and Bax. We also found that Myc reduces the expression of Bcl-2 and Bcl-xL and that the apoptosis-inducing stimuli up-regulate Bax expression. These results suggest that up-regulation of mtCLIC, together with a reduction in Bcl-2 and Bcl-xL, sensitizes Myc-expressing cells to the proapoptotic action of Bax.

Myc-induced tumors in mice suggested the roles of the p19ARF-p53 pathway and Bcl-2/Bcl-xL/Bax pathway in Myc-induced apoptosis; the lymphomas arising in Emu-myc transgenic mice frequently display inactivation of p19ARF-p53 pathway and overexpression of Bcl-2 (6, 13). Enforced expression of Bcl-2 or Bcl-xL or loss of Bax dramatically accelerates tumorigenesis in Myc transgenics (14–16). Myc-induced apoptosis requires Myc DNA-binding function, dimerization with Max, and its N-terminal transcriptional activation domain (17, 18), which is consistent with the notion that Myc induces apoptosis by modulation of target gene expression. Nevertheless, only a small number of Myc target genes have been implicated in apoptosis, and the precise mechanism of how Myc induces apoptosis still largely remains an enigma.

Apoptosis involves changes in the expression levels, subcellular locations, and modifications of key regulatory proteins. We therefore undertook a quantitative proteomic analysis of Myc-induced apoptosis. This analysis demonstrated the induction of a mitochondrial chloride ion channel, mtCLIC/CLIC4, by Myc in addition to protein changes already known to occur during apoptosis. mtCLIC has been previously identified as a key player in p53-induced apoptosis, and we show here that mtCLIC is also a mediator of Myc-induced apoptosis.

MATERIALS AND METHODS

Isotope-Coded Affinity Tag (iCAT) Reagent Labeling and Electrospray-Ionization Tandem Mass Spectrometry—Chromatin-enriched fractions (2.5 mg of each), soluble fractions (2.5 mg of each), and crude mitochondrial fractions (250 μg of each) were prepared as described (19, 20), dissolved in the iCAT labeling buffer (0.05% SDS, 6 M urea, 200 mM Tris (pH 8.3), and 5 mM EDTA), reduced with 5 mM Tris (2-carboxyethyl) phosphine for 30 min at 37 °C, and labeled (Rat1/vector cell lysate, isotopically light iCAT reagent; Rat1/Myc cell lysate, isotopically heavy iCAT reagent). The two labeled fractions were combined, proteolyzed to peptides with trypsin, and fractionated by cation-exchange chromatography. iCAT reagent-labeled peptides were purified using the biotin tag present in the reagent and analyzed by microcapillary high performance liquid chromatography-tandem mass spectrometry as described (19, 21–23). Tandem mass spectra were searched against the NCBI rat/mouse/human protein data base. Peptide/protein identification was validated by Peptide/ProteinProphet software tools (24, 25). Peptide and protein abundance ratios were calculated using ASAPRatio software tool (26).

Immunoblotting—The indicated amounts of cell lysates were separated by SDS-PAGE and were analyzed by immunoblotting as described...
(23). Anti-mtCLIC rabbit polyclonal antibody was described (27). Anti-c-Myc (sc-764), anti-Mad (sc-222), anti-p53 (sc-6243), anti-actin (sc-1616), and anti-PARP (sc-7150) antibodies were obtained from Santa Cruz Biotechnology. Anti-Bcl-2, Bcl-xL, and Bax antibodies were purchased from Cell Signaling Technology. Anti-FLAG M2 mouse monoclonal antibody was purchased from Sigma.

Reverse Transcription-PCR—Total cellular RNA was prepared with TRIzol reagent (Invitrogen) and was reverse-transcribed with M-MLV reverse transcriptase. The primers used for PCR amplification were: rat mtCLIC, 5’-CCT CAT CGA GCT TTT CGT CAA-3’ and 5’-TCT GGG TGT TTT GGT GAG AGC-3’; rat Max, 5’-ATG AGC GAT AAC GAT GAC ATC GA-3’ and 5’-TTA GCT GCC CTC CAT CCG CAG-3’. The amplification of the PCR products was monitored to assure that the PCR was within the range of linearity.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed as described (28). The primers used for PCR amplification were: mtCLIC upstream Myc site, 5’-CAG TAA TTA GCC CCA CCC AC-3’ and 5’-GCC AGC CCA AAG TAA GTT GCT C-3’; CDK4, 5’-CTC TGG GTG GCC TAG GTT G-3’ and 5’-TAG AGA GGC CCC CTC ACC-3’; β-globin, 5’-ATC TTC CTC CCA CAG CTC CT-3’ and 5’-TTT GCA GCC TCA GCC TCA CCT TCT TT-3’. PCR reactions were repeated using varying cycle numbers and different amounts of templates to ensure that results were within the linear range of PCR.

RESULTS

Quantitative Proteomic Analysis of Myc-induced Apoptosis—Myc induces apoptosis in a number of cell types under stress conditions. Rat1
mtCLIC/CLIC4 Mediates Myc-induced Apoptosis

fibroblasts are particularly sensitive to Myc-induced apoptosis and have been frequently employed as a model system (17, 20, 31). Using Rat1 cells, we performed a quantitative proteomic analysis of Myc-induced apoptosis upon serum withdrawal. We prepared mitochondrial (250 μg), chromatin (2.5 mg), and soluble (2.5 mg) fractions of Rat1 fibroblasts transduced with Myc-expressing retroviruses (Rat1/Myc) and Rat1 fibroblasts transduced with retroviruses not expressing Myc (Rat1/vector) after culturing in the absence of serum for 12 h. At 12 h after serum withdrawal, Rat1/Myc cells undergo robust apoptosis, whereas Rat1/vector cells do not display any sign of apoptosis. The protein expression in each fraction was compared by ICAT reagent labeling and electrospray-ionization tandem mass spectrometry (19, 21–23). The resulting data set was subjected to statistical analysis (24, 25), and at a probability score of 0.5 or higher (corresponding to a false positive identification rate of 6%), 209 proteins in mitochondrial fractions, 158 proteins in chromatin fractions, and 464 proteins in soluble fractions were identified and quantified.

As summarized in Fig. 1, we have identified a number of protein changes known to occur during apoptosis such as increased reactive oxygen species (ROS) synthesis pathways, nuclear translocation of death effector domain containing DNA-binding protein (DEDD), and increased pro-endothelial monoocyte-activating polypeptide II (EMAP II). In addition, novel protein changes such as the induction of mtCLIC in mitochondria and increase of NQO1 (NAD(P)H:quinone oxidoreductase 1) were also observed. (A complete listing of the proteins identified and their fold change is deposited in the NCBI Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) and is accessible through GEO Series accession number GSE3159.)

Expression of pro-EMAP II is induced upon tissue apoptosis (32), and the cleaved C-terminal fragment (EMAP II) is secreted from the apoptosing cells, resulting in phagocyte chemotaxis and cell engulfment (33). The CD95 apoptosis pathway is required for Myc-induced apoptosis upon serum withdrawal (34). Upon CD95-mediated apoptosis, DEDD translocates from the cytoplasm to the nucleus (35), where it activates caspase-6 and inhibits RNA polymerase I transcription (36). Myc was shown to increase the synthesis of ROS in mouse fibroblasts, a process proposed to mediate Myc-induced apoptosis (37). Our proteomic analysis also detected protein changes that result in increased ROS synthesis or that occur as a consequence of increased ROS. For example, reduced expression of a mitochondrial-specific H2O2 scavenging enzyme, PRDX3 (Fig. 1), would cause increased ROS synthesis and sensitize cells to apoptosis (38). Increased expression of myotrophin (39) and reduced expression of Bcl10 (40) would result in reduced NF-κB activity and diminished manganese superoxide dismutase-mediated ROS elimination (37). NQO1 (Fig. 1) is induced by reactive oxygen species, stabilizes p53, and mediates apoptosis (41), although its involvement in Myc-induced apoptosis has not been described. Molecular chaperones (Fig. 1) are induced by a diverse array of stresses, including ROS, and protect cells from apoptosis (42). These protein changes can in principle contribute to Myc-induced apoptosis or be the consequences of Myc-induced apoptotic changes. Identification of a number of protein changes known to be associated with apoptosis validates our proteomics approach.

We also observed up-regulation of mtCLIC in mitochondria of apoptosing Rat1/Myc cells. mtCLIC is a member of the CLIC family of intracellular chloride ion channels and was recently implicated in the apoptotic response to p53 (27). mtCLIC associates with the inner mitochondrial membrane, and its overexpression reduces mitochondrial membrane potential, releases cytochrome c into the cytoplasm, activates caspases, and induces apoptosis (27, 43). mtCLIC expression is induced by p53 and tumor necrosis factor α (27, 44). Induction of mtCLIC by p53 is thought to occur through binding of p53 to its cognate binding sites in the mtCLIC promoter (Fig. 3A). The proapoptotic activity of mtCLIC and its connection to p53 suggested that mtCLIC may be a novel mediator of Myc-induced apoptosis.

Induction of mtCLIC Expression by Myc—We have confirmed the up-regulation of mtCLIC as a consequence of Myc expression in Rat1 cells by immunoblotting (Fig. 2A). We observed 2.6-fold up-regulation in our ICAT analysis and 2.2-fold up-regulation in our immunoblots. We have found that the induction of mtCLIC by Myc is independent of serum. Myc also induced mtCLIC protein expression in REF52 and Balb/c3T3 cells (Fig. 2B). Furthermore, we detected elevated mtCLIC protein expression in colon Colo320 and lung N417 cancer cell lines with amplified c-myc (Fig. 2C). These cancer cell lines harbor p53 mutations, which may abrogate Myc-induced apoptosis. As expected, a Myc antagonist, Mad, reduced mtCLIC protein expression (Fig. 2D). Reverse transcription-PCR analysis demonstrated that Myc induces mtCLIC mRNA expression in Rat1 cells (Fig. 2E), indicating that up-regulation of mtCLIC by Myc occurs at least in part at the transcriptional level. Since mtCLIC is known to be induced by p53 (27, 44), it was of interest to test whether Myc can induce mtCLIC expression independently of p53. We thus introduced Myc or empty vector to p53-null Saos-2 cells and analyzed the level of mtCLIC. As shown in Fig. 2F, Myc induced mtCLIC expression in p53-null Saos-2 cells.

We next analyzed the mtCLIC gene promoter and found two possible Myc-binding sites, one canonical (CAGCGTG) site at −1000 and one non-canonical site at −3340 relative to the transcription start site (Fig. 3A). Employing a chromatin immunoprecipitation assay, we detected the binding of Myc to the downstream canonical Myc site but not the upstream non-canonical site (Fig. 3B). To determine whether Myc activates the mtCLIC promoter, we used the mtCLIC promoter linked to luciferase in transient transfection assays and found that Myc activates
the mtCLIC promoter transcription (Fig. 3C). Furthermore, activation of mtCLIC by Myc requires its heterodimerization/DNA-binding domain (basic-helix-loop-helix-zipper domain) (Fig. 3C). These results indicate that mtCLIC is a direct transcriptional target of Myc. Importantly, although Myc or p53 each activates the mtCLIC promoter modestly, when Myc and p53 are coexpressed, they display synergistic activation of the mtCLIC promoter (Fig. 3D).

Role of mtCLIC in Myc-induced Apoptosis—To assess the role of mtCLIC in Myc-induced apoptosis, we used several RNAi constructs to suppress the expression of mtCLIC in Rat1/Myc cells and analyzed apoptosis induction upon serum withdrawal. Retrovirus-mediated RNAi against mtCLIC (si-1) inhibited mtCLIC expression but did not affect Myc expression (Fig. 4A). The introduction of Myc or RNAi constructs did not affect p53 expression in Rat1 cells (Fig. 4A). si-1 did not affect the expression of other CLIC family members such as CLIC1. Upon serum withdrawal, Rat1/Myc cells underwent robust apoptosis. However, RNAi inhibition of mtCLIC prevented cell death by 60% as assessed by trypan blue exclusion assay (Fig. 4B). Furthermore, the cleavage of PARP was also inhibited (Fig. 4C). (Note that the PARP protein level is increased in apoptosing Rat1/Myc cells when compared with Rat1/vector cells (Fig. 1.).) Introduction of an mtCLIC mismatch RNAi construct, si-1-mut, which did not inhibit mtCLIC expression (Fig. 4A), failed to prevent apoptosis (Fig. 4, B and C). The second mtCLIC RNAi construct (si-2), targeting a distinct region of mtCLIC, also prevented PARP cleavage upon serum withdrawal, indicating the specificity of RNAi (Fig. 4D).

Myc also sensitizes cells to apoptosis upon DNA damage or glucose deprivation (45, 46), and we were interested in whether mtCLIC plays a role in these types of apoptosis. As shown in Fig. 5, mtCLIC RNAi also prevented Myc-induced apoptosis upon DNA damage caused by mitomycin C treatment or glucose deprivation, suggesting that mtCLIC plays a general role in mediating Myc-induced apoptosis.

Previous studies have demonstrated that Myc induces an activity that cooperates with Bax in apoptosis (31, 47), but the nature of this activity remains unknown. Thus, we examined whether induction of mtCLIC by Myc is required for cooperation with Bax in apoptosis. Rat1/vector cells and Rat1/Myc cells do not display any sign of apoptosis under normal culture conditions without any treatment. Transfection of FLAG-Bax induced modest cell death in Rat1/vector cells, and Rat1/Myc cells were hypersensitive to Bax-induced apoptosis (Fig. 6, A–C). mtCLIC RNAi inhibited this cooperative apoptosis induction by Myc and Bax, suggesting that mtCLIC supplies the Bax-cooperating activity induced by Myc. Based on these observations, we propose that mtCLIC is a critical mediator of Myc-induced apoptosis, which cooperates with Bax in apoptosis induction. Since mtCLIC RNAi did not completely prevent Myc-induced apoptosis, it is likely that Myc has other effector(s) for apoptosis.

To gain insight into this, we analyzed the expression of Bcl-2, Bcl-xL, and Bax upon serum withdrawal, DNA damage, or glucose deprivation. As shown in Fig. 7, Bcl-2 and Bcl-xL protein expression was significantly lower in Rat1/Myc cells than in Rat1/vector cells, and their expression remained lower in Rat1/Myc cells upon three different stimuli. Furthermore, these three stimuli induced the expression of Bax. Therefore, we
propose that Myc sensitizes cells to apoptosis by several pathways (see “Discussion”).

DISCUSSION

Our ICAT proteomic analysis identified mtCLIC as a protein, the expression of which is induced by Myc, and further analyses demonstrated that mtCLIC is a direct transcriptional target of Myc and is a critical mediator of Myc-induced apoptosis. There is evidence that mtCLIC expression is regulated at both transcriptional and post-transcriptional levels (27), and it is possible that Myc contributes to both levels of regulation, perhaps explaining why we readily detected mtCLIC in our proteomic analysis, whereas it was not identified in earlier expression studies.

The identification of mtCLIC as a critical Myc target mediating apoptosis induction is noteworthy because there has been only scant evidence concerning the nature of Myc targets involved in apoptosis. One important pathway of Myc-induced apoptosis appears to involve the release of cytochrome c from mitochondria (20), but how Myc causes cytochrome c release remains a mystery. A previous mRNA microarray analysis identified induction of several genes encoding mitochondrial proteins, including cyclophilin and a heat shock protein, by Myc, but their functions in the context of apoptosis have not been explored (48). Other Myc targets such as ornithine decarboxylase, CDC25A, and lactate dehydrogenase A have also been implicated in Myc-induced apoptosis (46, 49, 50), but no direct link to mitochondrial function has been demonstrated under apoptotic conditions. mtCLIC associates with the inner mitochondrial membrane, and its overexpression reduces mitochondrial membrane potential, resulting in release of cytochrome c into the cytoplasm, activation of caspases, and induction of apoptosis (27, 43). mtCLIC was previously shown to cooperate with Bax in apoptosis induction, although direct physical interaction of mtCLIC and Bax was not detected (27). The location to mitochondrial inner membrane and the putative pore-forming and ion transport activities of mtCLIC (51) are consistent with a role for the opening of mitochondrial membrane channels in the apoptotic response. It was also reported that mtCLIC levels are elevated in mitochondria isolated from mtDNA-depleted cells and that these mitochondria incorporate more 36Cl− than parental mitochondria (52). mtCLIC may mediate apoptosis by modulating chloride ion transport through the mitochondrial membrane.

The results presented here placed mtCLIC at an intersection of the two apoptosis pathways induced by Myc and p53. The p53 pathway was shown to be an important mediator of Myc-induced apoptosis (5, 53, 54). It was presumed that Myc is upstream of p53 in apoptotic signaling, activating p53 through p19ARF. Downstream mediators of p53-dependent apoptosis such as Bax (15, 47) and Puma (55, 64) were shown to play roles in Myc-induced apoptosis. However, Myc is also known to induce p53-independent apoptosis by some unknown mechanism (56, 57). The activation of the mtCLIC promoter by Myc and p53 suggests a new mechanism in which these two apoptosis inducers work together to modulate the expression of a proapoptotic mitochondrial chloride ion channel. Activation of p53 by DNA damage induces either cell cycle arrest or apoptosis, and Myc was shown to be a principal determinant of the outcome of p53 response (58–60). Myc shifts the p53 response from cell cycle arrest to apoptosis. Although the inhibition of p21 expression through Myc-Miz-1 interaction was proposed as an underlying mechanism (58, 59), cooperative activation of mtCLIC transcription by Myc and p53 may also contribute to the switch from arrest to apoptosis.

The effect of Myc and p53 on mtCLIC expression may vary depending on the cell types or culture conditions. p53 expression is not induced in Rat1/Myc cells when compared with Rat1/vector cells (Fig. 4A), suggesting that p53 does not account for the sensitization to apoptosis in
Thus, p53 does not appear to play a role in modulating mtCLIC expression during apoptosis of Rat1/Myc cells. However, in cells induced to apoptosis by overexpression of p53, there was a robust induction of mtCLIC expression and antisense inhibition of mtCLIC expression prevented p53-induced apoptosis (27). Thus, the contribution of Myc and p53 to the regulation of mtCLIC expression seems to be context-dependent.

We have found that in Rat1 cells, mtCLIC is induced by Myc regardless of the presence of serum, DNA damage, or glucose. We presume that mtCLIC up-regulation sensitizes cells to apoptosis and that serum withdrawal as well as DNA damage or glucose deprivation triggers the execution of apoptosis. Since mtCLIC RNAi did not completely prevent Myc-induced apoptosis, we analyzed the expression of Bcl-2, Bcl-xL, and Bax in Rat1/vector and Rat1/Myc cells upon different stimuli (Fig. 7). We found that Myc reduces the expression of Bcl-2 and Bcl-xL regardless of the stimuli. These findings are consistent with a previous report demonstrating that Myc reduces Bcl-2 and Bcl-xL expression in mouse embryonic fibroblasts and B cells (64). In addition, we observed that the apoptosis-inducing stimuli such as serum withdrawal, DNA damage, and glucose deprivation up-regulate Bax expression. Based on these observations, we propose that 1) up-regulation of mtCLIC and 2) down-regulation of Bcl-2 and Bcl-xL sensitize Myc-expressing cells to apoptosis and that up-regulation of Bax by apoptosis-inducing stimuli triggers the execution of apoptosis.

The human mtCLIC gene maps to chromosome 1p36.11, which displays loss of heterozygosity in 90% of N-myc-amplified neuroblastomas (i.e. one mtCLIC allele is deleted) (61). Introduction of intact chromosome 1 or 1p into N-myc-amplified neuroblastomas resulted in cessation of proliferation and cell death (62, 63), suggesting the presence of a proapoptotic tumor suppressor in 1p36. mtCLIC may function as a tumor suppressor, which is inactivated by genetic alteration (deletion and possibly point mutation) in 1p36.11 region. Indeed, mtCLIC transcript levels are significantly reduced in human tumors from multiple different tissues.4 Our findings suggest that mtCLIC may be a key element in the apoptotic response to multiple signals.

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FIGURE 6. mtCLIC mediates cooperative apoptosis induction by Myc and Bax. A, expression of FLAG-Bax. Rat1 cells were transfected with FLAG-Bax expression vector, and the expression of FLAG-Bax was examined 24 h after transfection by anti-FLAG immunoblotting using 15 μg of each cell lysate. The untransfected Rat1/vector cells serve as a negative control. B, Rat1/Myc cells are hypersensitive to Bax-induced cell death, and mtCLIC RNAi abolishes this hypersensitivity. The fraction of trypan blue-stained cells was determined 24 h after FLAG-Bax transfection. V, empty vector; C, mtCLIC RNAi prevents PARP cleavage induced by Myc and Bax. The arrow and arrowhead denote the full-length and cleaved PARP, respectively.

FIGURE 7. Expression of Bcl-2, Bcl-xL, and Bax in Rat1/vector and Rat1/Myc cells upon different apoptosis-inducing stimuli. Rat1/vector and Rat1/Myc cells were deprived of serum or glucose or treated with 5 μM mitomycin C for the indicated times (in hours), and temporal profiles of Bcl-2, Bcl-xL, and Bax protein expression were determined by immunoblotting using 15 μg of each cell lysate. V, empty vector.

Y. Shiio, unpublished data.
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