Targeting Bcl-2 stability to sensitize cells harboring oncogenic ras

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ABSTRACT

The pro-survival factor Bcl-2 and its family members are critical determinants of the threshold of the susceptibility of cells to apoptosis. Studies are shown that cells harboring an oncogenic ras were extremely sensitive to the inhibition of protein kinase C (PKC) and Bcl-2 could antagonize this apoptotic process. However, it remains unrevealed how Bcl-2 is being regulated in this apoptotic process. In this study, we investigate the role of Bcl-2 stability in sensitizing the cells harboring oncogenic K-ras to apoptosis triggered by PKC inhibitor GO6976. We demonstrated that Bcl-2 in Swiss3T3 cells ectopically expressing or murine lung cancer LKR cells harboring K-ras rapidly underwent ubiquitin-dependent proteasome pathway after the treatment of GO6976, accompanied with induction of apoptosis. In this process, Bcl-2 formed the complex with Keap-1 and Cul3. The mutation of serine-17 and deletion of BH-2 or 4 was required for Bcl-2 ubiquitination and degradation, which elevate the signal threshold for the induction of apoptosis in the cells following PKC inhibition. Thus, Bcl-2 appears an attractive target for the induction of apoptosis by PKC inhibition in cancer cells expressing oncogenic K-ras.

INTRODUCTION

Bcl-2 family consists of members that are able either to promote cell survival (such as Bcl-2 and Bcl-XL) or apoptosis (for example, Bax or Bak) [1, 2]. Levels or balances between the pro-and anti-apoptotic factors determine thresholds of cells for the induction of apoptosis. Bcl-2 resides in the endoplasmic reticulum and mitochondria [3, 4]. Through binding to Bax or regulating the membrane permeability of the mitochondria, Bcl-2 was shown to interfere with releasing of the mitochondrial apoptotic factors (for example, Bax or cytochrome c) to the cytosol, and thereby suppressed cell death process [3, 5, 6]. Studies also showed that increases of the expression of Bcl-2 have correlated well with the development of human malignancies, for example, in lung cancer [7–10]. Furthermore, in vitro and in vivo experiments also demonstrated that a significant change of Bcl-2 in tumorigenesis, inflammatory process, heart failure or other diseases [11–15]. However, the mechanisms by which Bcl-2 is being regulated and further affects the susceptibility of cells to apoptosis are not fully understood yet.

The protein degradation is a key event in the regulation of various functions or activities of cells [16–18]. The ubiquitination-proteasome pathway is one of the major protein degradation pathways and proven to timely controls the amount of the expression of proteins involved in the regulation of each critical cellular activity, such as cell cycle checkpoints, tumor
surveillances, cellular or DNA damage repair and duration of intracellular signal transduction. In the process of ubiquitination, protein degradation is triggered by E1 ubiquitin activating enzyme. Subsequently, E2 ubiquitin-conjugating enzymes transfer ubiquitin from E1 to E3 to catalyze substrate ubiquitination [19, 20]. Proteins are covalently bound to ubiquitin that is a polypeptide with 76 amino acids and ubiquitously expressed in cells [16–18]. Ubiquitin-tagged proteins eventually are recognized by the proteasome and being degraded, in which lysine residue appears critical [21, 22].

Keap1 is an adaptor protein and participates in Cul3-mediated degradation of Nrf2 during oxidative or radiation-induced stresses [18, 23–25]. Functioning as a sensor for cellular stresses, Keap1 has been shown to be critical for the inhibition of Nrf2 and Bcl-2 activities [26–28]. Keap1 conjugates with the Cul3-containing E3 ubiquitin ligase and mediates Nrf2 and Bcl-2 for their ubiquitination and degradation. The degradation of Bcl-2 in TNF-α or staurosporin-stimulated cells was reported to be through the ubiquitination [29, 30]. Upon the stimulations, Bcl-2 was interacted with Keap1 and then being rapidly degraded, leading to dramatically sensitizing the cells to TNF-α or staurosporin-induced apoptosis.

Bcl-2 exerts its function through affecting the membrane potential of the mitochondria and activation of caspases [31, 32]. Bcl-2 proteins share 4 sequence homology domains (BH 1–4). Through BH 1–4 domains, Bcl-2 members are able to form homodimers and heterodimers in order to influence the susceptibility of cells to apoptosis. The expression levels of Bcl-2 have been demonstrated to be a crucial factor in cancer progression and development (33, 34). Bcl-2 expression was also proven to play a potential role in inflammatory reactions [11, 35]. Studies indicated that Bcl-2 interacted with Keap1 and further being degraded through Keap1-mediated ubiquitination [26–28]. The interference of Bcl-2 degradation promoted cell survival, suggesting the importance of this protein modulation process in the regulation of cell fate.

This study aimed at getting insights into how Bcl-2 is being regulated in the cancerous cells expressing oncogenic K-ras after the treatment of PKC inhibitor. We demonstrated that Bcl-2 degradation appeared to be involved in apoptosis triggered by GO6976 (an inhibitor specific for PKC α and β) or shRNA-PKC α plus β in Swiss3T3 cells transformed by v-K-ras or murine lung cancer LKR cells harboring oncogenic K-ras. In this apoptotic process, Bcl-2 rapidly bound to Keap1 and Cul3 for being ubiquitinated and further degraded. Using bcl-2 mutants mutated at several lysine sites or deletion mutants at BH 1–4 domains, we showed that the lysine-17 and BH-2 of this pro-survival factor are crucial for the ubiquitination. Our study suggested that Bcl-2 is a crucial target for sensitizing cancer cells expressing oncogenic K-ras after being treated with the PKC inhibitor.

RESULTS

Bcl-2 is important for sensitizing the cells expressing oncogenic K-ras to apoptosis triggered by GO6976

Oncogenic Ras is able not only promote cell growth or differentiation, but also function as a pro-apoptotic factor in sensitizing various types of cancer cells under certain circumstances (such as the abrogate of PKC) [36, 37]. To test whether mutated K-ras could transmit the apoptotic signaling in our experimental setting, murine fibroblasts Swiss3T3, SK1 (Swiss3T3 cells stably transfected with v-ras), murine Lung epithelial LA4 and LKR (that were isolated from the lung neoplasia foci of the K-ras transgenic mouse) cells were infected with sc, shRNA-bcl-2, or wt-bcl-2, prior to the treatment of GO6976 (a specific PKC α and β inhibitor) that was proven to induced apoptosis as efficient as co-knockdown by shRNAα and β under our experimental setting [37]. Subsequently, Annexin V assay was conducted (Fig 1A). After the treatments, only a few of Swiss3T3 or LA4 cells were apoptotic. In comparison, more than 35% of SK1 or LKR cells underwent apoptosis. The overexpression of wt-bcl-2 moderately suppressed the magnitude of apoptosis in the cells expressing oncogenic K-ras, following the addition of GO6976. The knockdown of bcl-2 significantly increased the susceptibility of SK1 and LKR cells to apoptosis induced by GO6976. The results suggested that Bcl-2 is a rate limiting factor in this apoptotic process.

The correlation between Bcl-2 expression and the resistance to the induction of apoptosis has been reported [38, 39]. In response to growth-related stimulation, Bcl-2 was shown to be upregulated and further desensitize cancer cells to apoptosis [38, 40, 41]. Therefore, the expression of Bcl-2 was examined in the cells after being treated with GO6976 for 48 h when the occurrence of apoptosis became evident (Fig 1B, left panels) and the amounts of the expression were measured (Fig 1B, right panel). Bcl-2 expression in the treated cells were decreased, in comparing with that in untreated cells, which further supported the notion that Bcl-2 is an important factor in this apoptotic process.

Bcl-2 degradation and ubiquitination were accelerated in the treated cells expressing oncogenic K-ras

Bcl-2 is a crucial factor in the regulation of the sensitivity of cell to apoptotic stimulation. The overexpression or knockdown of bcl-2 changed the response of the cells harboring oncogenic K-ras to GO6976 (Fig 1A) and the expression of this pro-survival factor in the cells was also altered upon GO6976 treatment (Fig 1B). It led us to explore the importance of Bcl-2 stability in our experimental setting. RT-PCR (Real-Time PCR)
analysis was performed to test whether the change of Bcl-2 expression in this apoptotic process was at the transcriptional level. The kinetics of the gene expression of \( bcl-2 \) in the cells expressing oncogenic \( K-ras \) with or without GO6976 treatment was similar, suggested that the transcriptional machinery was not involved in the regulation of Bcl-2 expression in this apoptotic process (data not shown). Subsequently, the protein stability of Bcl-2 was examined by protein pulse-chasing analysis (Fig 2A). Cell lysates from GO6976-treated or untreated cells were prepared at various time points after adding CHX to block the synthesis of proteins and then subjected to immunoblotting. The kinetics of the degradation of Bcl-2 in GO6976-treated SK1 and LKR cells was much faster than that in untreated cells. The addition of the PKC inhibitor had no a significant effect on Bcl-2 stability in Swiss3T3 or LA4 cells. Thus, the data indicated that the protein stability of Bcl-2 played a significant role in this apoptotic process in an oncogenic K-Ras-dependent fashion.

Ubiquitination is one of important mechanisms for protein degradation. In this process, lysine residues of proteins are covalently conjugated with ubiquitins, prior to being degraded. Bcl-2 was reported to undergo ubiquitination for its degradation [26–30]. Bcl-2 ubiquitination in SK1 or LKR with or without GO6976 treatment was tested by immunoprecipitation using anti-Bcl-2 antibody and immunoblotting with anti-ubiquitin antibody (Fig 2B, left panel). The reciprocal experiment was also performed (Fig 2B, right panel). Bcl-2 was rapidly conjugated with ubiquitin after the addition of the PKC inhibitor, which was not occurred in the untreated cells. The data further suggested that GO6976 treatment accelerated Bcl-2 degradation, which mitigated the anti-apoptotic activities in the cells expressing oncogenic \( K-ras \).

Lysine-17 is critical for Bcl-2 ubiquitination and degradation

Bcl-2 undergoes the ubiquitin-proteasomal pathway for its degradation [26–30]. In the first step of this degradation process, lysine residues of proteins are being targeted by the proteasome complexes [21, 22]. The amino acid sequence analysis revealed there are 4 lysine residues existing at the positions of 17, 22, 214 and 236 in Bcl-2 [26, 27]. The residue of lysine 17 was reported to be responsible for Bcl-2 ubiquitination [26, 27]. To test the role of lysine 17 in our experimental setting, \( bcl-2 \) mutant with the mutation of the amino acid 17 to alanine (R17) and \( wt-bcl-2 \) were introduced into SK1 cells. Subsequently, the degradation of Bcl-2/R17 or Bcl-2 was analyzed in the cells treated with GO6976. The degradation of Bcl-2/R17 was significantly delayed. At 6 h after the addition of cycloheximide (CHX), a moderate amount of Bcl-2 could be detected in GO6976-treated cells (Fig 3A). As expected, wt-Bcl-2 was rapidly degraded in SK1 cells after the treatment. To determine that Bcl-2 degradation was through ubiquitination, the cell lysates were extracted from untreated or treated SK1 cells transfected with \( wt-bcl-2 \) or \( bcl-2/R17 \) vector tagged with \( GST \). Immunoprecipitation was performed with

![Figure 1: Effect of Bcl-2 on the onset of apoptosis induced by GO6976.](image)
anti-GST antibody and immunoblotting with anti-ubiquitin antibody (Fig 3B). The fragmented Bcl-2 conjugated with ubiquitins were present in GO6976-treated SK1/wt-bcl-2 cells, which was absent in the untreated cells or GO6976-treated SK1/bcl-2/R17 cells. The result was obtain from the reciprocal experiment. The bcl-2 mutants at the residue of lysine 22, 214 or 236 that was mutated to alanine were also introduced into the cells under the same experimental condition. The degradation patterns of the protein products of the mutants were similar as that of wt-Bcl-2 (data not shown). The data suggested the lysine-17 of Bcl-2 appears a potential target in regulating the susceptibility of cells harboring oncogenic ras to apoptosis in the absence of PKC.

BH2 or 4 domain of Bcl-2 regulates Bcl-2 stability in GO6976-induced apoptosis

Bcl-2 contains 4 conserved homology domains (BH1–4), each of which interacts with pro- or anti-apoptotic factor, and further positively or negatively regulate apoptosis [42, 43]. The BH1–4 deletion mutants of bcl-2 were constructed and introduced in SK1 cells, respectively (Fig 3C). After GO6976 treatment, the stability of Bcl-2 with different BH deletions was tested (Fig 3D). The exogenous Bcl-2 with the BH2 or 4 deletion, but not the BH1 or 3 deletion, was unable to be ubiquitinated in SK1 cells treated with GO6976. The data again suggested that the lysine 17 and BH2 domain of Bcl-2 are important for its ubiquitination and subsequent degradation. The inability of the BH4 deletion mutant to be degraded might be due to the lack of the lysine 17.

Formation of the ubiquitin complexes is perturbed by R17, A2 or A4 mutant of bcl-2

Keap1, a member of the BTB-Kelch family, serves as an adaptor protein for Cul3-dependent ubiquitination. The interaction of Keap1 with Bcl-2 was shown to promote Bcl-2 ubiquitination and degradation [26, 27]. Therefore, the influences of Bcl-2 mutation and deletion on its association with Keap1 in our experimental setting were tested. After introduced bcl-2 deletion mutants (Δ1, Δ2 or Δ4) or R17 into SK1 cells, respectively, the co-immunoprecipitation with anti-GST antibody and immunoblotting with anti-Bcl-2 antibody (Fig 3E). The exogenous Bcl-2 with the BH2 or 4 deletion, but not the BH1 or 3 deletion, was unable to be ubiquitinated in SK1 cells treated with GO6976. The data again suggested that the lysine 17 and BH2 domain of Bcl-2 are important for its ubiquitination and subsequent degradation. The inability of the BH4 deletion mutant to be degraded might be due to the lack of the lysine 17.
Figure 3: Importance of the lysine 17, BH2 or 4 in Bcl-2 degradation. A. bcl-2 mutant with the lysine 17 mutation (R17) or wt-bcl-2 was constructed in the pEX-GST expression vector and then transfected in SK1 cells. After the treatment of GO6976, the degradation of the mutant Bcl-2/R17 or wt-Bcl-2 was examined by immunoblotting using anti-GST antibody. The blot was re-probed with anti β-actin antibody to determine equal loading of total proteins per lane. B. Co-IP and immunoblotting of wt-Bcl-2 or Bcl-2/R17 with Ubiquitin in SK1 cells and the reciprocal co-IP/immunoblotting were tested. C. Schematic diagram of Bcl-2. The locations of BH domains are presented by grey boxes. D. bcl-2 mutants with various BH deletions in the pEX-GST expression vector were transfected into SK1 cells. The transfectants were treated with GO6976 and lystates were prepared. The kinetics of the deletion mutant proteins were tested as described above. E. Co-IP and immunoblotting of Bcl-2 deletion mutants and Ubiquitin in SK1 cells and the reciprocal co-IP/immunoblotting were tested.
treated with GO6976. The results also suggested that BH2 domain was another critical element for the formation of the E3 ligase complexes and, further for promoting Bcl-2 ubiquitination and degradation.

**R17, Δ2 or Δ4 mutant of Bcl-2 desensitizes the cells to apoptosis**

To further test the influence of Bcl-2 mutants on the induction of apoptosis mediated by GO6976, Annexin V assay was conducted. After introduced wt- and bcl-2 mutants, approximately 30% of SK1 cells expressing bcl-2/R22, R214 or R236 mutants underwent apoptosis after GO6976 treatment (Fig 5A, left panel). The magnitude of apoptosis in the treated SK1 cells overexpressing these mutants was attenuated in comparison with the treated parental cells, and comparable with the cells overexpressing wt-bcl-2. The deletion mutants of bcl-2 were also transfected into SK1 cells. The ectopic expression levels of wt- or mu-Bcl-2 were also quantitated (Fig 5A, right panel). The magnitude of apoptosis in response to GO6976 treatment was similar in the cells overexpressing wt-, Δ1- or Δ3-bcl-2 (Fig 5B, left panel). The amounts of the expression of the deletion mutants were analyzed (Fig 5B, right panel). Consistently,
the overexpression of Δ2- or Δ4-bcl-2 significantly reduced the magnitude of GO6976-induced apoptosis in SK1 cells. Together, the data implicated the importance of Bcl-2 stability in sensitizing SK1 cells to apoptosis after the inhibition of PKC. In this apoptotic process, lysine 17 and BH2 domain appeared critical in the regulation of Bcl-2 degradation.

**DISCUSSION**

Active mutations of ras can be detected in more than 30% of human malignancies, which has drawn intensive attentions and efforts to understand the structures, biochemistry and biology of this onco-protein, and further to target it as new strategies for treating tumors harboring mutated ras. The notion of the therapeutic approaches is that in order to maintain and cope with a high metabolic rate of a cancer cell, oncoproteins need the supports from parallel to or distal of signaling pathways. Disruption of the supporting signaling would perturb adapted homeostasis in cancer cells and further trigger an apoptotic crisis. PKC has been shown to promote tumor growth and cooperate with oncogenic Ras in tumorigenesis [43, 44]. Studies demonstrated that cancer cells harboring oncogenic ras were proven to be sensitive to the inhibition of PKC for the induction of apoptosis [36, 45]. In this process, Bcl-2 appeared to play a counteractive role [45, 46]. In this study, we thoroughly investigated the role of Bcl-2 in this apoptotic process. We demonstrated that the cells harboring oncogenic K-ras became more sensitive to the PKC inhibitor when bcl-2 was knocked down and less responsive upon bcl-2 overexpression. In this apoptotic process, Bcl-2 was strongly ubiquitinated and rapidly degraded, resulting in sensitizing the cells to apoptosis. Our study suggested that PKC cooperates with oncogenic K-Ras to promote or support the survival of cancer cells, probably via increasing Bcl-2 stability.

The ubiquitin-proteolytic mechanism is an important system for the degradation of some proteins in mammalian cells. In dying cells, levels of ubiquitinated proteins were observed to be increased, implicating the connection between protein degradation and apoptotic processes [47, 48]. The function of Bcl-2 was shown to be controlled at the stages of the transcription, posttranscriptional modifications and degradation [42, 43]. In TNFα-mediated apoptosis, Bcl-2 was ubiquitinated and rapidly degraded in human endothelial cells [29]. Furthermore, Bcl-2 became unstable in human lung and kidney cancer cells upon UV-light exposure [26]. Upon nicotine treatment, it was demonstrated that via activating the mitogenic signaling pathways, Bcl-2 ubiquitination and degradation processes were attenuated, which facilitated the establishment of the chemo-resistance in lung cancer cells [28]. Therefore, the proteolytic process regulating Bcl-2 stability appears one of the key players in sensitizing cancer cells expressing oncogenic ras to apoptosis.

Keap1 plays a crucial role in regulating the expression of Nfr2, Bcl-2 and other proteins and further cellular activities [23–25]. In antioxidant response, Keap1 was demonstrated to be one of the components in the E3 ubiquitin ligase complex, which controls ubiquitination and proteasomal degradation of the transcriptional factor Nrf2. In TNFα-induced apoptosis, MAPK/ERK1/2 signaling was suggested to be responsible for Bcl-2 activation [29]. Due to the interaction with Keap1, the negative effect of Bcl-2 on Bax was lifted, which further sensitized cells to apoptosis [26]. The treatment with MAPK inhibitor blocked Bcl-2 phosphorylation and subsequently triggered the degradation of this pro-survival factor. We previously demonstrated that nicotine treatment delayed Bcl-2 dephosphorylation and further increased its stability, which caused Bcl-2 to be unable to associate with Keap1 and delayed to be degraded. In this study using GO6976-induced apoptosis, we further proved the importance of Keap1 in regulating apoptosis involved in Bcl-2. Keap1 appears, through controlling Bcl-2 expression levels, to function as a molecular sensor to regulate apoptosis in cancer cells harboring oncogenic ras.

One of the mechanisms by which Bcl-2 exerts its pro-survival function is through its BH (Bcl-2 homology) 1–4 domains [42, 43]. Some apoptotic factors, such as Bax and Bik, also possess BH domains. Through BH domains, Bcl-2 associates with pro-apoptotic factors and regulates the fate of a cell. The stability of Bcl-2 was shown to influence the susceptibility of cells to apoptosis [29]. Upon the exposure of UV light, the lysine 17 was demonstrated to be critical for the ubiquitination of this pro-survival factor to apoptosis [26]. In our experimental setting, Bcl-2, once its lysine 17 was mutated or deleted, was unable to be degraded in the cells expressing oncogenic ras after PKC suppression. Interestingly, Bcl-2 became very stable when its BH2 domain that does not contain lysine residues, was deleted. It is possible the BH2 domain serves to anchor one of the components of Cul3-containing E3 ubiquitin ligase and is necessary for Bcl-2 degradation. The further investigation of the role of the BH2 domain of Bcl-2 is under way.

In summary, we have demonstrated that Bcl-2 ubiquitination and degradation play an important role in sensitizing cancer or transformed cells expressing oncogenic ras to apoptosis triggered by PKC inhibition. Our study also identified that impairment of Bcl-2 by mutating its lysine 17 or deleting BH2 domain prolonged the half-life of this pro-survival factor and further desensitized the cells to apoptosis induced by GO6976. Thus, Bcl-2 dictates cell viability and death not only through its heterodimerization with apoptotic factors, but also being controlled by Keap1/Cul3-mediated ubiquitination. Dysregulation of Keap1 or Cul3 function would definitely affect cell survival involving Bcl-2.
MATERIALS AND METHODS

Cell lines and treatments

Swiss3T3 and LA4 cells were obtained from ATCC (Manassas, VA, USA). LKR murine lung cells are generous gift from Dr. Jacks (MIT, Cambridge) and have been used in various studies [49–51]. The cells were cultured in DMEM medium containing 10% fetal calf serum. SK1 cells are Swiss3T3 cells stably transfected with v-K-ras and maintained in the growth medium containing G418 (200 μg/ml). GO6976 was purchased from Sigma. Bcl-2 mutants were inserted in the pEX-GST expression vector and obtained from Dr. Luo (Boston University).

Annexin V-FITC apoptosis detection assay

After treatments, cells were harvested and stained with Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) according to manufacturer’s instructions and subsequently, analyzed by a flow cytometer.

Immunoprecipitation and immunoblotting analyses

For immunoprecipitation, after treatments, cell lysates were isolated and immunoprecipitated with an antibody. The precipitates were then separated on a SDS-PAGE gel for immunoblotting (see below).

For immunoblotting, lysates were isolated from treated cells and separated by SDS-PAGE gels. Following the transfer, nitrocelluloses were incubated with the designated primary antibodies overnight at 4°C, and subsequently incubated with second antibodies for 2 h at room temperature. The blots were finally detected by chemiluminescence.

Statistical analysis

Three to five independent repeats were conducted in all experiments. Error bars represent these repeats. A Student’s T test was used and a p value of < 0.05 was considered significant.

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CONFLICTS OF INTEREST

There is no financial conflict of interest concerning this study.

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