Dynamics of Bcl-2Protein Phosphorylation: A Mini Review

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Abstract

The regulation of apoptosis depends upon the Bcl-2 protein family. The process of cell death and survival is highly complicated and regulated by various types of extrinsic as well as intrinsic network of biological system. Several enzymes and regulators play crucial role in cell death and survival cycle not only in healthy but also in pathological state particularly in cancer. In cancerous cells, various proto-oncogenes and anti-apoptotic proteins are activated and responsible for the cell survival and longevity. The mechanism of activation and inactivation of various proteins in cell survival is regulated by the process of phosphorylation (kinases) and dephosphorylation (phosphatases). The current review will summarize the dynamics of Bcl-2 phosphorylation and its role in apoptosis and cell survival.

Keywords: apoptosis, cancer, phosphorylation, kinases, Bcl-2

Introduction

Apoptosis or cellular suicide is a genetically programmed process that is important for recycling of damaged cells, tissue homeostasis and embryonic development. The activation mechanism of apoptosis requires extrinsic and intrinsic pathways where various enzymes including cysteine proteases called as "caspases" play a crucial role (Poreba *et al*., 2019). The activation of caspases at molecular level depends upon the phosphorylation (Parrish *et al*., 2013). In extrinsic pathway, activated death receptors like tumor necrosis factor receptor (TNFR), TNF-related apoptosis-induced ligand (TRAIL) and first apoptosis signal (Fas) recruits the initiator caspases (caspase8 and caspase10) (Wang and El-Deiry, 2003; Tummers and Green, 2017). Contrarily, in intrinsic pathway, stress releases cytochrome c from mitochondria leading to the formation of a complex called "apoptosome" that in turn activate caspase 9 (initiator caspase)which recruit caspase 3 (executioner caspase) in the same way asin extrinsic pathway (Parrish *et al*., 2013).

Apoptosis is regulated positively by pro-apoptotic protein Bax and negatively by anti-apoptotic Bcl-2 family proteins both containing Bcl-2homology (BH) domain (Chipuk and Green, 2008; Brunelle and Letai, 2009). Bcl-2 not only reverses the effect of Bax but also halts the release of cytochrome c from mitochondria (Greenhalf *et al*., 1996; Gross *et al*., 2000). Activation/inactivation of pro/anti-apoptotic proteins depends upon the process of

phosphorylation in which phosphate group from ATP is transferred to one of the amino acid residues of serine, tyrosine or threonine residues by protein kinases (PKs). Imbalance between anti- and pro-apoptotic proteins may lead to inability to respond appropriately to apoptotic stimuli and growth of malignant cells and ultimately cancer that is why cancerous cells do not respond well to treatment (Petros *et al*., 2004).

In biological system, the regulation of various cellular processes such as signal transduction pathways, apoptosis, cell cycle and growth depend upon proteins. After translation, protein undergoes various changes collectively called post-translational modifications (PTMs) (Hill et al., 2019). These modifications include glycosylation, nitrosylation, methylation, ubiquitination, acetylation, proteolysis, lipidation as well as most important phosphorylation (main topic of the current review). These modifications are not only associated with normal cell's physiology but also the pathological state. Understanding the role of PTMs is very critical for the investigation of disease prevention and treatment (Wang *et al*., 2014; Zhao and Jensen, 2009).

Phosphorylation

Phosphorylation and dephosphorylation are two important biological processes catalyzed by protein kinases (PKs) and phosphatases that add or remove a phosphate group of proteins respectively. Study of these momentous processes helps in understanding of not only human health but also the pathological aspects of various disorders including cancer (Seternes et al., 2019). Unique and complete balancing of phosphorylated proteins within a biological system defines its phosphoproteome which is more dynamic than its proteome (Franck *et al*., 2015).

Phosphorylation acts as a molecular switch to turn the protein in on/off state (Ardito, Fatima, et al. 2017; Colin *et al*., 2008). Under the influence of phosphorylation three crucial aspects such as interactions, degradation and location control of proteins have been investigated. About one third of proteins undergo phosphorylation and majority is phosphorylated at multiple sites in a single protein. Most common residues are serine and threonine amino acids whereas other amino acids such as tyrosine, glutamic acid, aspartic acid and histidine are also subjected to phosphorylation (Puttick *et al*., 2008; Hunter, 2009). Higher magnitude of tyrosine phosphorylation has been observed in activated platelets (Ferrell and Martin, 1988). However, most of the cells exhibit highest level of phosphorylation on serine residue and threonine amino acid.

Bcl-2 family proteins

The Bcl-2 family protein members and contain one to four functional homology domains (BH1 to BH4) (Petros *et al*., 2004). Interestingly, Mcl-1 and Bcl-xL show anti-apoptotic role similar to Bcl-2 protein, whereas Bad, Bid and Bax show pro-apoptotic activity. Several members of anti-apoptotic proteins contain all four BH regions including Ced-9, Bcl-xL, Bcl-w and Bcl-2. Moreover, BHRF1 protein, KSHV-Bcl-2 and Mcl-1 exhibit strong homology sequence in three BH regions including BH1, BH2 and BH3 domains. BH3 region of pro-apoptotic proteins exhibit interaction with anti-apoptotic members and are responsible for promoting programmed cell death (Letai*et al*., 2002; Kvansakul and Hinds, 2013).

The 3D structure of Bcl-2 family members exhibit two central α-helices (hydrophobic in nature) that are surrounded by six or seven amphipathic α-helices having varying lengths. Interestingly, a prominent hydrophobic groove can be observed on the surface of anti-apoptotic proteins and the residues of this groove are the binding sites for various pro-apoptotic proteins like Bad and Bax containing peptides that mimic BH3 region. Several Bcl-2 family proteins contain a carboxy-terminal hydrophobic region that is accepted for mitochondrial membrane localization. Moreover, membrane localization behavior of anti- and pro-apoptotic proteins is not similar. Bcl-2 and Bcl-xL are localized at cytoplasmic surface of mitochondrial outer membrane; anchoring of these proteins may play a critical role associated with the integrity of mitochondria. Pro-apoptotic members that are present in cytoplasm only localize to outer membrane of mitochondria when they get activated (Petros *et al*., 2004).

Bcl-2 protein undergoes several types of phosphorylation. One important type occurs during mitosis in un-stressed cells, involving a small proportion of Bcl-2 (Choi and Zhu, 2019). Moreover, investigations by researchers revealed that Thr56, CDK1/cyclin B and JNK were found to be associated with phosphorylation of Bcl-2 protein in mitosis (Yamamoto *et al*., 1999; Furukawa *et al*., 2000; Du *et al*., 2004). Cells treated with anti-mitotic drugs undergo stress which lead to high levels of phosphorylation as observed in all Bcl-2 members and several kinases are involved (Table 1).

Table 1: Various kinases involve in Bcl-2 phosphorylation

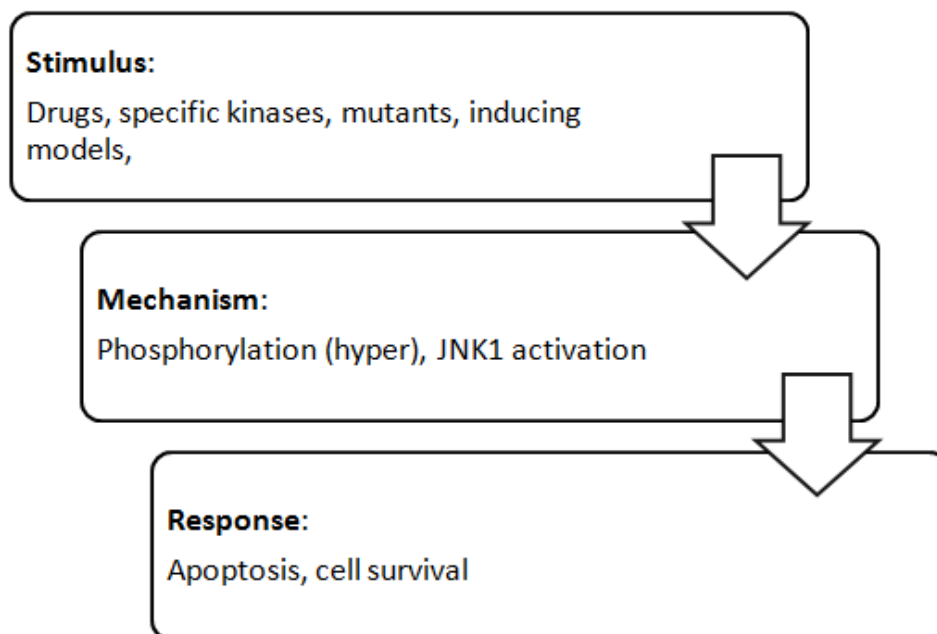|	Name of kinases	References
1	Raf-1	Balgosklonny *et al*., 1997
2	PKA	Srivastava *et al*., 1998
3	PKC	Ito *et al*., 1997; Ruvolo *et al*., 1998
4	MAPK	Breitschopf *et al*., 2000
5	JNK/SAPK	Maundrell *et al*., 1997
6	CDK1	Terrano *et al*., 2010
7	CDK6	Ojala *et al*., 2000
8	ASK/JNK	Srivastava *et al*., 1999

Phosphorylation of Bcl-2 protein

Under the influence of apoptotic stimuli, Bcl-2 protein performs survival function through inhibition of cytochrome c release from mitochondria. Multiple sites within the Bcl-2 protein have been phosphorylated including Serine at position (70 and 87) and Threonine (56 and 75). Phosphorylation of these sites may be a marker for mitotic cell division as well as targets for ASK/MKK7/JNK1 pathway in anti-cancer therapies (Park *et al*., 2019).

Bcl-2 phosphorylation in response to a range of treatments is specific to stimuli as well as cell type which lead to the activation or inactivation of anti-apoptotic behavior (Table 2; Figure 1). Role of Bcl-2also depends upon mitochondria, movement of ions and small molecules necessary for induction of cell death. Bcl-2 is present in mitochondria, endoplasmic reticulum and nuclear envelope (Krajewski *et al*., 1993; Popgeorgiev *et al*., 2018). Association of Bcl-2 with nuclear matrix is reflecting its important feature in genomic organization (Wang *et al*., 1999).

Table 2: Bcl-2 phosphorylation, apoptosis and cell survival

Stimulus	Response	Mechanism	Reference
Paclitaxel (2hrs)	Apoptosis	Phosphorylation of Bcl2 (Thr69, Ser70, Ser87) and was not exclusive for Ser70	Yamamoto *et al.*, 1999 Haldar*et al.*, 1995
Paclitaxel	Apoptosis	Bcl-2 phosphorylation without PKC/MAPKs	Blagosklonny *et al.*, 1997; Blagosklonny *et al.*, 1999
Glucocorticoid induction in T-lymphocytes	Apoptosis	(Thr56, Thr74, Ser70, Ser87) → Thr56 and Ser87 are required for Bcl-2 to protect cells from glucocorticoid-induced apoptosis	Huang *et al.*, 2002
Nutrient starvation	Apoptosis	JNK1 activation cause multisite phosphorylation (Thr69, Ser70, Ser87) of Bcl-2 and dissociation of Bcl-2-Beclin1 complex	Wei *et al.*, 2008
Multisite phosphomimetic mutants	Apoptosis	Phosphorylation → (Thr69E*, Ser70E, Ser87E) → fails to co-immuno precipitate with Beclin1 → fails to inhibit autophagy → all done due to activated JNK1 *E is gultamic acid	Wei *et al.*, 2008
Ca^{2+} conc. in ER	Apoptosis	Bcl-2 phosphorylation → no binding to both BH3-only and multi-domain pro-apoptotic proteins → dissociation of complexes	Bassik *et al.*, 2004
Cellular starvation	Apoptosis	autophagy promoted as cell survival mechanism	Levine and Klionsky, 2004
Tumor necrosis factor (TNF)	Apoptosis	Prolonged JNK1 activation	Ventura *et al.*, 2006
IL3-withdrawal	Apoptosis	Bcl-2 phosphorylation is inhibited	Ito *et al.*, 1997
p38 MAPK	Apoptosis	NGF-deprivation, TNF, NO → multisite Bcl-2 phosphorylation → pro-apoptotic events →	Torcia *et al.*, 2001; Rosini *et al.*, 2000;

		release of cytochrome c → in both cellular system and cell free experiments	Rosini *et al*., 2004; Ishikawa *et al*., 2003; Tamura *et al*., 2000
p38 MAPK	Cell survival	Bcl-2 phosphorylation → specific targets (Thr56 and Ser87) → alter anti-apoptotic potential of Bcl-2	De Chiara *et al*., 2006
Specific kinases	Cell survival	Bcl-2 phosphorylation decreased anti-apoptotic function	Srivastava *et al*., 1999; Fan *et al*., 2000; Torcia *et al*., 2001; De Chiara *et al*., 2006
Nutrient starvation	Cell survival	Alanine mutants of Bcl2 (Thr69Ala*, Ser70Ala, Ser87Ala) lead to binding of Bcl-2-Beclin1 complex	Wei *et al*., 2008
IL3-induced model	Cell survival	Bcl-2 phosphorylation lead to increased anti-apoptotic potential	Ito *et al*., 1997
Ginsenoside-Rh2 induction	Cell survival	Transient JNK1 activation	Ham *et al*., 2003
Taxol-induced model	Cell survival	hyperphosphorylation of various kinases → including Raf-1 kinase → Bcl-2 phosphorylation → strong inhibition of anti-apoptotic potential	Blagosklonny*et al*., 1996

Stimulus:
Drugs, specific kinases, mutants, inducing models,

Mechanism:
Phosphorylation (hyper), JNK1 activation

Response:
Apoptosis, cell survival

Figure 1 Overview of Phosphorylation, apoptosis and cell survival

Glucocorticoid-induced apoptosis and Bcl-2

Mitochondria are involved in steroid-induced apoptosis which depends largely upon the phosphorylation of Bcl-2 protein. Cell shrinkage appears to be important feature of apoptosis (Huang and Cidlowski, 2002). It has been observed that Ser70 mutation did not abolish protective effect of Bcl-2 against dexamethasone-induced shrinkage, whereas mutation at Thr56, and 74 and Ser87 abolished the potential of Bcl-2 to inhibit steroid induced cell shrinkage.

Anti-apoptotic behavior of Bcl-2 is inhibited during glucocorticoid-induced apoptosis of T-lymphocytes under the influence of mutation at Thr56 or Ser87 (Huang and Cidlowski, 2002). In lymphoid cells, apoptosis is induced by glucocorticoids whereas, over-expression of Bcl-2 block apoptosis induced by glucocorticoids. Huang and Cidlowski (2002) prepared five transfected Bcl-2 cell lines of WEHI 7.1 cells expressing either alanine mutants of Thr56, Thr74, Ser70 or Ser87. Mutation at Thr56 and Ser87 completely abolished the anti-apoptotic property of Bcl-2 whereas mutation at Ser70 did not alter its ability to block glucocorticoid-induced apoptosis. Interestingly, Thr74 mutation partially disturbed the role of Bcl-2 (Huang and Cidlowski, 2002).

JNK and IL-3 induced phosphorylation of Bcl-2

Anti-apoptotic role of Bcl-2 may be enhanced by JNK and/or IL-3 induced phosphorylation at Ser70in murine myeloid cells lacking growth factor (Deng *et al*., 2001;May *et al*., 1994).Phosphorylation of Bcl-2 was associated with increased cell survival upon the addition of IL-3 in chemotherapeutic-induced (drug etoposide) apoptosis (Ruvolo *et al*., 2001). Bryostatin-1 induced vigorous Bcl-2 phosphorylation and promotes cell survival by acting as a strong protein kinase C (PKC) agonist and found alternate for IL-3 in cell survival. PKC was observed to be specific Bcl-2 kinase responsible for its phosphorylation in the same serine residues both *in vivo* and *in vitro* experiments. Seven common phosphorylation sites were found in human and murine Bcl-2 at serine 24, 70, 102, 158, 164, 202 and 213. Moreover, it was investigated that only Ser70 mutation makes the Bcl-2 negative anti-apoptotic in its function reflecting physiological relevance of Bcl-2 phosphorylation at Ser70 (Kennelly and Krebs, 1991; May *et al*., 1994; Ito *et al*., 1997).

JNK mediated phosphorylation of Bcl-2

JNK mediated phosphorylation of Bcl-2 protein is associated not only with programmed cell death but also with autophagy. JNK mediated phosphorylation has been observed to interfere with binding of Bcl-2 to pro-apoptotic BH3 domain-containing proteins such as Bax and pro-autophagy BH3 domain-containing proteins such as Beclin-1 (Wei *et al*., 2008). Kinetic relationship and correlation of Bcl-2 with Bax and Beclin-1, caspase-3 activation as well as Bcl-2 phosphorylation was investigated during nutrient starvation (Wei *et al*., 2008). The nutrient conditions are responsible to regulate the binding of Bcl-2 and Beclin-1 proteins (Pattingre *et al*., 2005). This binding was minimum during nutrient stress and maximum when the nutrients were in excess. Dissociation of Beclin-1 from Bcl-2 during starvation implicates phosphorylation of Bcl-2 by stress-activated signaling molecule, JNK1 (Wei *et al*., 2008). During nutrient deprivation, activated JNK induces phosphorylation at multiple sites of Thr69, Ser70 and Ser87 in the non-structured loop of Bcl-2 (located between BH3 and BH4 domains).

Alanine (Thr69Ala, Ser70Ala and Ser87Ala) and non-phosphorylatable Bcl2 mutants, eliminates the starvation-induced separation of Bcl-2 and Beclin-1 and halts autophagy. While, Bcl-2 phosphomimetic mutant (Thr69Glu, Ser70Glu and Ser87Glu) failed to co-immuno-precipitate with Beclin-1 and no autophagy inhibitory activity was observed indicate that JNK mediated multiple site phosphorylation of Bcl-2 activates its separation from Beclin-1 to induce

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autophagy (Wei *et al*., 2008). Moreover, Bcl-2 phosphorylation suppresses its binding to both BH3-only and multi-domain pro-apoptotic proteins and encourages apoptosis (Bassik *et al*., 2004). It has been reported that autophagy is activated as a cell survival mechanism during nutrient deprivation (Levine and Klionsky, 2004), while delayed cellular nutrient withdrawal finally leads to apoptosis. In association with the mechanism of autophagy, temporary JNK activation encourages cell survival in contrast to prolonged JNK activation mediates cell death (Ventura *et al*., 2006; Ham *et al.,* 2003).

Sphingolipid-ceramide and phosphorylation of Bcl-2

Sphingolipid-ceramide is well known for its apoptogenic behavior by playing crucial role in signal transduction pathways and in cellular stress (Jarvis *et al*., 1996). Ceramide can activate mitochondrial protein phosphatase (PP2A) which is responsible for apoptosis via dephosphorylation of Bcl-2, phosphorylated Bcl-2 is responsible for cell survival. Ceramide is also responsible for activation of ceramide-activated protein phosphatase (CAPP). However, dynamic phosphorylation/dephosphorylation of Bcl-2 may act as survival sensor during stress stimuli (Dobrowsky *et al*., 1993; Westwick *et al*., 1995). Interestingly, Bcl-2 protein has been observed to undergo phosphorylation at various amino acid residues (multiple site phosphorylation) reflecting both apoptotic and anti-apoptotic role (Ruvolo *et al*., 2001).

Conclusion

Bcl-2 phosphorylation in response to a range of treatments is specific to stimuli as well as cell type. Phosphorylation sites for Bcl-2 protein identified by investigators are most commonly observed at serine (70 and 87), and threonine (56, 69, 74 and 75). Moreover, transient activation of JNK1 leads to the cell survival while prolonged JNK1 activation leads to cell death reflecting dual role of Bcl-2 protein due to phosphorylation.

Conflict of interest

The authors declare no conflict of interest.

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