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Alpha4 enhances the phosphorylation of Bcl-xL induced by c-jun N-terminal kinase (JNK)

Kano Tanabe[1], Kazuki Takemoto[1], Akihiro Goto[1], Ryutaro Kajihara[2], Hideyuki Yamamoto[3], Seiji Inui[1]*

[1]Department of Immunology and Hematology, Graduate School of Health Sciences, Faculty of Life Sciences, Kumamoto University, (JAPAN)

[2]Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, (JAPAN)

[3]Department of Biochemistry, Graduate School of Medicine, University of the Ryukyus, (JAPAN)

E-mail: inui@kumamoto-u.ac.jp

ABSTRACT

c-Jun N-terminal kinase (JNK) phosphorylates Ser62 of Bcl-xL to induce degradation by the ubiquitin proteasome pathway in WEHI-231 cells upon BCR crosslinking. To elucidate the mechanism underlying this regulation of Bcl-xL phosphorylation, we prepared a system in which JNK phosphorylated Bcl-xL in HEK293T cells. This phosphorylation was Ser62-specific, because the phosphorylation of the Ser62Ala (S62A) mutant form of Bcl-xL was not observed with a pSer62-specific antibody. We found that a signal transduction molecule,alpha4, enhanced the phosphorylation of Bcl-xL by JNK. Alpha4 associated both with JNK and Bcl-xL, and was mainly localized in cytoplasm. Therefore, alpha4 may regulate the activity of JNK toward Bcl-xL in cytoplasm. ! 2014 Trade Science Inc. - INDIA

KEYWORDS

Bcl-xL;
JNK, alpha4;
Apoptosis;
Phosphorylation.

INTRODUCTION

Bcl-xL is one of the anti-apoptotic members of the Bcl-2 family[1]. Bcl-xL functions in apoptosis involving the mitochondrial pathway, including BCR crosslinking-induced apoptosis of WEHI-231 cells[1, 2]. We previously showed that BCR crosslinking induced Ser62 phosphorylation and the subsequent degradation of Bcl-xL by the ubiquitin proteasome pathway in WEHI-231 cells[3].

ERK, JNK and p38 are members of the MAPK family[4]. Many stimuli, including cytokines and stress, activate JNK[5]. JNK is important in stress-induced apoptosis mediated by the mitochondrial pathway[5], and JNK-deficient cells showed severe impairment in stress-induced apoptosis[5]. The activation of the JNK pathway results in either cell survival or apoptosis, depending on the stimulus and the cellular context[5]. JNK is important in the induction of apoptosis in lymphocytes[6]. However, it remains elusive howJNK induces apoptosis. JNK activation may cause apoptosis by both transcription-dependent and -independent mechanisms[5]. One ofthe proposed mechanisms of transcription-independent control is that JNK phosphorylates Bcl-2 family members to regulate apoptosis[5]. Sustained activation of JNK results in apoptosis, while transient activation results in cell survival[7]. In WEHI-231 cells, BCR crosslinking induced sustained activation of JNK[8].

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Protein phosphatase 6 (PP6) is a serine/threonine phosphatase which belongs to the PP2A family[9]. The yeast homolog of PP6, Sit4, was identified as a cell cycle regulator[10],and human PP6 was shown to be an apoptosis-related molecule involved in tumor development[11]. We also showed that PP6 is important for the regulation ofapoptosis[12]. PP6 is composed of a catalytic subunit and regulatory molecules, the SAPs[13].

Alpha4 was originally identified as a molecule associated with the BCR complex[14]. Alpha4 is ubiquitously expressed[14], and its sequence is highly conserved among mammalian species[15]. It was later revealed that alpha4 associated with the catalytic subunits of PP2A, PP4 and PP6[16-19]. Alpha4 regulates the enzymatic activity of PP2A and PP6[18, 20]. Gene targeting experiments showed that alpha4 was essential for cell survival[21, 22]. Recently, other functions of alpha4 have been reported[23, 24].

Here, we show that JNK phosphorylates Ser62 of Bcl-xL in HEK293T cells, and this phosphorylation is enhanced by alpha4, with alpha4 associating with both JNK and Bcl-xL. An analysis of the subcellular localization revealed that alpha4 may regulate the phosphorylation of Bcl-xL by JNK in the cytoplasm.

MATERIALS AND METHODS

Cells and reagents

The anti-Bcl-xL antibody (Ab) (Abcam, Cambridge, MA), anti-Bcl-xL (pS62) phosphospecific polyclonal Ab (Millipore, Bedford, MA), and anti-JNK Ab (Cell Signaling Technology, Danvers, MA), were purchased for Western blot and/or immunoprecipitation studies. The anti-β-tubulin III and anti-histone H3 antibodies were purchased from Sigma chemical company (St. Louis, MO) and Active Motif (Carlsbad, CA), respectively. Anti-alpha4 was prepared as describedpreviously[18]. M41 was a kind gift from Dr. Rolink at the University of Basel (Basel, Switzerland). The JNK inhibitor, SP600125,and siRNA against human alpha4 were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Santa Cruz biotechnology (Dallas, TX), respectively. The HEK293T cell line was as described previously[3].

Plasmid DNAs and transfection

The wild type and S62A mutant forms of Bcl-xL cDNAs were described in a previous study[3]. Human alpha4 cDNA was described previously[18], and was subcloned into the pCMV-Tag2 vector (Stratagene, La Jolla, CA). Human JNK1 cDNA was a kind gift from Dr. Ogata at Mie University in Japan. Transfection was performed using HilyMax transfection reagent (Dojindo, Kumamoto, Japan), as describedpreviously[3]. Transfection of siRNAs was done with RNAiMAX (Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

Subcellular fractionation

The cytosolic, nuclear, and crude mitochondrial fractions were obtained from fresh rat brain by the method described byde Robertis et al.[25]. The mitochondrial fraction was prepared after osmotic shock of the crude mitochondrial fraction.

Immunoprecipitation and Western blot analyses

The immunoprecipitation and Western blot analyseswere performed as describedpreviously[3]. The lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 15 mM NaPPi, 100 mM β-glycerophosphate, 25 mM NaF, 100 μM Na_3VO_4, 0.5 % CHAPS) with phosphatase inhibitor cocktail (Nacalaitesque, Kyoto, Japan) was used for the analysis of the co-immunoprecipitation of alpha4 with other molecules.

RESULTS

Alpha4enhances the phosphorylation of Ser62 of Bcl-xL induced by JNK

To elucidate the mechanism underlying theregulation of Bcl-xL phosphorylation JNK, we performed and*in vivo* phosphorylation assay with HEK293T cellstransfected with JNK and Bcl-xL cDNAs. In the presence of JNK, Bcl-xL was phosphorylated in HEK293T cells (Figure 1A).

This phosphorylation of Bcl-xL was confirmed to be mediated by JNK, because the addition of a JNK inhibitor, SP600125,prevented it (Figure 1A). We also transfected the S62A mutant form of Bcl-xL cDNA into HEK293T cells with JNK cDNA. As shown in Figure 1B, an anti-phsopho-Ser62 Ab did not detect any signal in the lysates of S62A cDNA-transfected cells. We previously showed that PP6 negatively regulated the

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phosphorylation of Bcl-xL in WEHI-231 cells following BCRcrosslinking[3]. PP6 is composed of the catalytic subunit, PP6c, and regulatory subunits[13], but the expression levelsof associated molecules (PPR1, PPR2, and PPR3) were very low in WEHI-231 cells (data not shown). Therefore, we tested the effect of another PP6 regulatory molecule,alpha4[16 to19], on the phosphorylation of Bcl-xL in HEK293T cells. The addition of alpha4 enhanced the phosphorylation of Bcl-xL by JNK (Figure 1C). To study the effects of alpha4 gene knockdown on the phosphorylation of Bcl-xL by JNK, we utilized a siRNA against alpha4. When alpha4 siRNA was transfected to suppress the expression of alpha4 in HEK293T cells, the phosphorylation of Bcl-xL was decreased as shown in Figure 1D.

Alpha4 associates with both JNK and Bcl-xL in the cytoplasmic compartment

Considering the possibility that alpha4 may associate with JNK or Bcl-xL, we studied the association of alpha4 with JNK and Bcl-xL. Co-immunoprecipitation experiment indeed showed that alpha4 associated with both JNK and Bcl-xL Figure 2A.

This suggested that alpha4; Bcl-xL, and JNK interact in the same subcellular compartment. Because there was a report that alpha4 resides in the nucleus[23], we investigated the subcellular localization of these three molecules.Cytoplasmic, nuclear, and mitochondrial fractions were prepared from rat brain as described in the Materials and Methods section. Bcl-xL was found in all three compartments, as reported previously Figure 2B[1]. JNK was found most abundantly in the cytoplasm and was also found in the nucleus and mitochondria at moderate levels. Finally, alpha4 was detected mostly in the cytoplasmic fraction, and was barely detectable in the nucleus or mitochondria. A Western blot analysis with an anti-histone H3 Ab revealed that no nuclear fraction was contaminated with mitochondria or cytoplasm. A Western blot analysis with an anti-β tubulin IIIAb suggested that the cytoplasmic fraction hadslightly leaked into the nuclear and mitochondrial

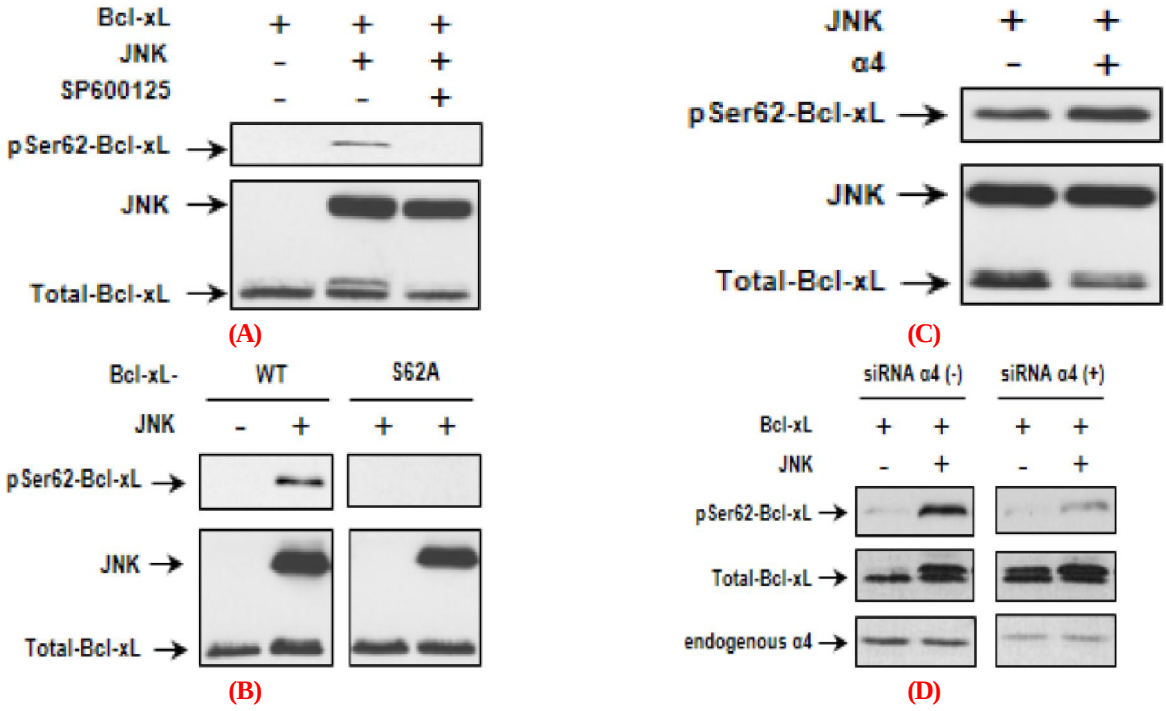

Figure 1 : Alpha4 regulates the phosphorylation of Ser62 of Bcl-xL by JNK; (A) JNK1 and Bcl-xL cDNAs were transfected into HEK293T cells as indicated, in the presence or absence of SP600125. The lysates were prepared after 24 h of transfection and were subjected to a Western blot analysis with an anti-pSer62 specific Ab. (B) Wild type or S62A mutant cDNA of Bcl-xL was transfected together with JNK cDNA, and the level of phospho-Bcl-xL was detected as in (A). (C) Alpha4 had a regulatory function in the phosphorylation of Bcl-xL by JNK. The effects of the co-transfection of alpha4 cDNAwereexamined by a Western blot analysis with an anti-pSer62 specific Ab.(D) The phosphorylation of Bcl-xL by JNK was diminished in the presence of siRNA against alpha4. The expression level of alpha4 was decreased after transfection of the siRNA intoHEK293T cells.

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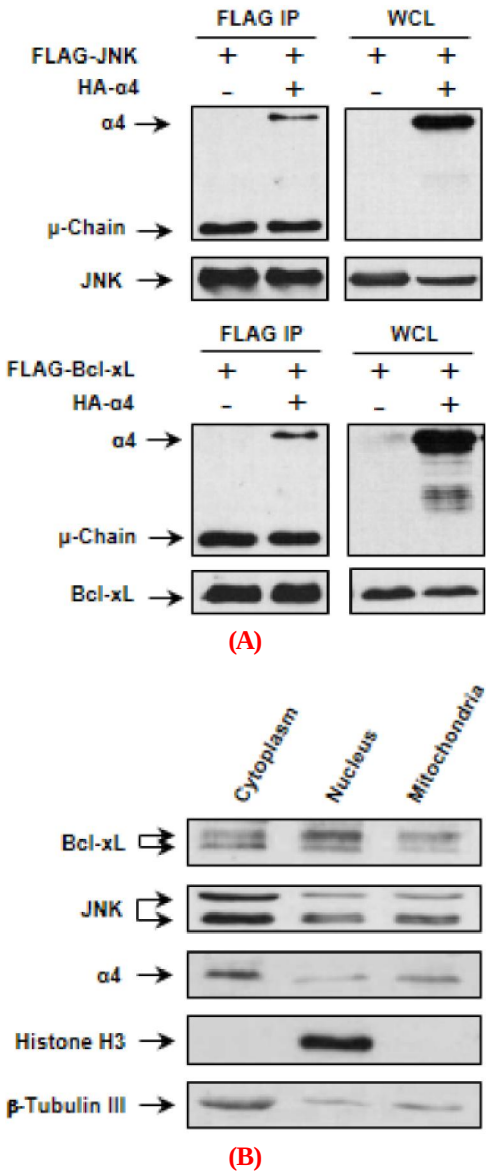

Figure 2 : Alpha4 associate swith both JNK and Bcl-xL in the cytoplasm; (A) FLAG-JNK1 or FLAG-Bcl-xLcDNA was transfected into HEK293T cells, together with HA-tagged alpha4 cDNA, as indicated in the figure. The association between alpha4 and JNK or Bcl-xL was assessed by immunoprecipitat ization of these molecules. Anti-β tubulin III and anti-histone H3 Abs were employed to verify the fractionation.

fractions. These results suggested that alpha4 may regulate the phosphorylation of Bcl-xL by JNK specifically in the cytoplasm.

DISCUSSION

JNK is necessary for the induction of apoptosis through the mitochondrial-dependent, but not independent,pathway[5]. UV-induced apoptosis is mitochondria-dependent and requires JNK, but this process does not require transcription[5]. JNK has at least 10 isoforms, and each isoform is believed to have unique functions[5, 26]. JNK1 is responsiblefor UV-induced apoptosis[26]. We here showed that JNK1 is important in the phosphorylation of Bcl-xL, which is important in the BCR-induced apoptosis.

We also showed that alpha4 regulated the activity of JNK toward Bcl-xL. However, it was not determined whether alpha4 works directly on JNK or not. JNK does not phosphorylate Bcl-xL *in vitro*[27], suggesting that other molecules are necessary for its activity on Bcl-xL. The activity of JNK is tightly regulated by upstream kinases, including MKK4/7, and MKKKs such as ASK1 and TAK1[5]. PP2C or PP6 regulates the MKKK-MKK-JNK pathway by dephosphorylating ASK1 or TAK1, respectively[28 to 30]. The elucidation of the mechanism by which alpha4 regulates the JNK activity needs further investigation.

Previous reports showed that alpha4 deficiency resulted in apoptosis[21, 22]. It was also reported that alpha4 did not associate with either JNK or Bcl-xL[22]. The difference between our present results and those in the previous studies may be explained by the difference in the cell employed for the experiments, or the fact that we used a different type of detergent that is suitable for detecting associationsbetween proteins. The phosphatase inhibitors which were included in the lysis buffer may also have helped to maintain the association between alpha4 and other molecules.

Alpha4 was originally identified as a component of the BCR complex, and cloning of its cDNA and the determination of its predicted amino acid sequence revealed that it had neither trans-membrane nor nuclear localization signals[14]. One report indicated that alpha4 resides mainly in the nucleus[23]. The differences between that study and our present findings may be explained by the different tissues/cells examined. However, the fact that the major binding partners, PP2A or PP6, have been reported to exist in the cytoplasm corroborates our results[31]. Bcl-xL was previously demonstrated to exist in both the cytoplasm and mitochondria under normal conditions, and to translocate from the cytoplasm to the mitochondrion uponthe induction ofapoptosis[1]. It was first reported that JNK was expressed mainly in the cytoplasm and translocated to the

nucleus to phosphorylate c-jun[5]. However,arecent report showed that JNK was localized in themitochondria[32]. JNK and alpha4 may regulate the protein level of Bcl-xL in the cytoplasm or in the mitochondrion upon the induction of apoptosis to tilt the fate of the cell to death by promoting the degradation of the Bcl-xL protein by the ubiquitin proteasome pathway.

REFERENCES

[1] R.J.Youle, A.Strasser; Nature Reviews Molecular CellBiology, **9**, 47-59 **(2008)**.
[2] T.L.Rothstein; Current Opinion in Immunology, **8**, 362-371 **(1996)**.
[3] R.Kajihara, H.Sakamoto, K.Tanabe, K.Takemoto, M.Tasaki, Y.Ando, S.Inui; Journal of Immunology, **192**, 5720-5729 **(2014)**.
[4] G.L.Johnson, R.Lapadat; Science, **298**, 1911-1912 **(2002)**.
[5] R.J.Davis; Cell, **103**, 239-252 **(2000)**.
[6] M.Rincón, A.Whitmarsh, D.D.Yang, L.Weiss, B.Dérijard, P.Jayaraj, R.J.Davis, R.A.Flavell; Journal of Experimental Medicine, **188**, 1817-1830 **(1998)**.
[7] S.Papa, F.Zazzeroni, C.G.Pham, C.Bubici, G.Franzoso; Journal of Cell Science, **117**, 5197-5208 **(2004)**.
[8] E.Tanaka, K.Hata, J.Mizuguchi; Experimental Cell Research, **312**, 3728-3738 **(2006)**.
[9] D.L.Brautigan; FEBS Journal, **280**, 324-345 **(2013)**.
[10] A.Sutton, D.Immanuel, K.T.Arndt; Molecular and Cellular Biology, **11**, 2133-2148 **(1991)**.
[11] J.P.MacKeigan, L.O.Murphy, J.Blenis; Nature Cell Biology, **7**, 591-600 **(2005)**.
[12] R.Kajihara, S.Fukushige, N.Shioda, K.Tanabe, K.Fukunaga, S.Inui; Biochemical and Biophysical Research Communications, **401**, 350-355 **(2010)**.
[13] B.Stefansson, D.L.Brautigan; Journal of Biological Chemistry, **281**, 22624-22634 **(2006)**.
[14] S.Inui, K.Kuwahara, J.Mizutani, K.Maeda, T.Kawai, H.Nakayasu, N.Sakaguchi;Journal of Immunology, **154**, 2714-2723 **(1995)**.
[15] K.Maeda, S.Inui, H.Tanaka, N.Sakaguchi; European Journal of Biochemistry, **264**, 702-706 **(1999)**.
[16] C.J.Di Como, K.T.Arndt; Genes and Development, **10**, 1904-1916 **(1996)**.
[17] K.Murata, J.Wu, D.L.Brautigan; Proceedings of the National Academy of Sciences of the United States of America, **94**, 10624-10629 **(1997)**.
[18] S.Inui, H.Sanjo, K.Maeda, H.Yamamoto, E.Miyamoto, N.Sakaguchi; Blood, **92**, 539-546 **(1998)**.
[19] J.Chen, R.T.Peterson, S.L.Schreiber; Biochemical and Biophysical Research Communications, **247**, 827-832 **(1998)**.
[20] T.D.Pickett, D.L.Brautigan; Journal of Biological Chemistry, **281**, 30503-30511 **(2006)**.
[21] S.Inui, K.Maeda, D.R.Hua, T.Yamashita, H.Yamamoto, E.Miyamoto, S.Aizawa, N.Sakaguchi; International Immunology, **14**, 177-187 **(2002)**.
[22] M.Kong, C.J.Fox, J.Mu, L.Solt, A.Xu, R.M.Cinalli, M.J.Birnbaum, T.Lindsten, C.B.Thompson; Science, **306**, 695-698 **(2004)**.
[23] W.J.McDonald, S.M.Sangster, L.D.Moffat, M.J.Michelle, J.Henderson, C.K.L.Too; Journal of Cellular Biochemistry, **110**, 1123-1129 **(2010)**.
[24] H.Du, Y.Huang, M.Zaghlula, E.Walters, T.C.Cox, M.A.Massiah; Journal of Biological Chemistry, **288**, 21341-21350 **(2013)**.
[25] E.De Robertis, G.R.De L.Arnaiz, L.Salganicoff, A.P.De Iraldi, L.M.Zieher; Journal of Neurochemistry, **10,** 225-235 **(1963)**.
[26] S.C.Dreskin, G.W.Thomas, S.N.Dale, L.E.Heasley; Journal of Immunology, **166**, 5646-5653 **(2001)**.
[27] L.Du, C.S.Lyle, T.C.Chambers; Oncogene, **24**, 107-117 **(2005)**.
[28] M.G.Li, K.Katsura, H.Nomiyama, K.Komaki, J.Ninomiya-Tsuji, K.Matsumoto, T.Kobayashi, S.Tamura; Journal of Biological Chemistry, **278**, 12013-12021 **(2003)**.
[29] T.Kajino, H.Ren, S.Iemura, T.Tatsume, B.Stefansson, D.L.Brautigan, K.Matsumoto, J.Ninomiya-Tsuji; Journal of Biological Chemistry, **281**, 39891-39896 **(2006)**.
[30] W.Min, Y.Lin, S.Tang, L.Yu, H.Zhang, T.Wan, T.Luhn, H.Fu, H.Chen; Circulation Research, **102**, 840-848 **(2008)**.
[31] B.Stefansson, D.L.Brautigan; Cell Cycle, **6**, 1386-1392 **(2007)**.
[32] Y.Zhao, T.Herdegen; Molecular and Cellular Neuroscience, **41**, 186-195 **(2009)**.